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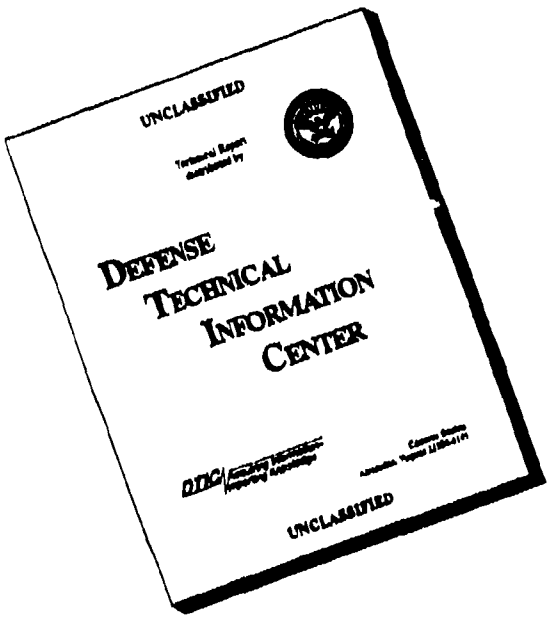

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Annual Report No. 2
Preformulation Studies on Antimalarials

Annual Progress Report

John L. Lach, Principal Investigator
Douglas R. Flanagan, Assistant Principal Investigator
Lloyd E. Matheson, Jr., Assistant Principal Investigator

July 1981

Supported by
U.S. Army Medical Research and
Development Command
Fort Detrick
Frederick, Maryland 21701

Contract No. DAMD 17-79-C-9136

College of Pharmacy
University of Iowa
Iowa City, Iowa 52242

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Antimalarials; preformulation; WR6026·2HCl; WR2975 phosphate; liposomes; stability; cobalt salts; WR171,669·HCl; PVP coprecipitates; dissolution rates; solubility

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

This annual report contains:

1. A description of liposome preparation techniques

2. Methods for evaluating liposome entrapment

3. UV spectral data on WR6026·2HCl

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4. Tonicity results on aqueous solutions of WR6026·2HCl and WR2975 phosphate (primaquine),

5. Entrapment results for WR6026·2HCl in various liposome formulations,

6. Entrapment results for WR2975 phosphate in liposomes,

7. Recommendations for biological evaluation of liposomes,

8. Solubility studies on WR6026·2HCl,

9. Stability studies in WR6026·2HCl in various aqueous media,

10. Cobalt salt studies

11. Coprecipitate studies of WR171,669·HCl with polyvinylpyrrolidone (PVP),

12. Production information on WR171,669·HCl (Halofantrine hydrochloride) capsules.

13. Quality control test results on the capsules produced.

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SUMMARY

This annual report represents preformulation and formulation studies conducted in the second year of this contract on the following drugs:

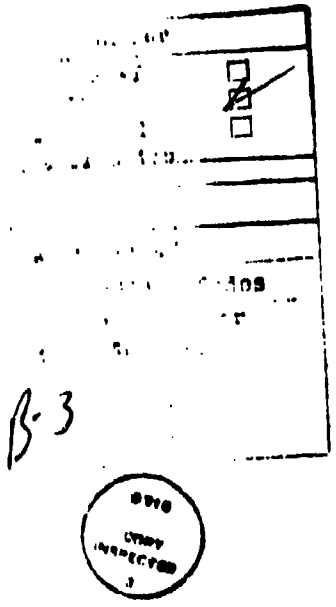
WR6026·2HCl

WR2975 Diphosphate (Primaquine)

WR171,669·HCl (Halofantrine)

Most of these studies centered on the development of liposome formulations of WR6026·2HCl and PVP coprecipitate formulations of WR171,669·HCl.

Studies were also conducted on the solution properties of cobalt salts to be used as antidotes for cyanide intoxication.



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INTRODUCTION

Liposome development studies on WR6026·2HCl were conducted in three stages.

Firstly, entrapment studies were conducted following procedures supplied by WRAIR (1) with concentrations of WR6026·2HCl that were low (i.e. around 1 mg/ml). Secondly, after determining the isotonic concentration of WR6026·2HCl (104 mg/ml), entrapment studies were conducted over a range of drug concentrations (5 mg/ml to 104 mg/ml). Thirdly, modifications were made in both the liposome preparation methods and lipid composition to optimize the entrapment characteristics of the liposomes.

LIPOSOME PREPARATION TECHNIQUES

Preparation 1 and 2[a]

The chemicals employed and their sources are shown in Table I. Liposomes were then prepared by procedures supplied by WRAIR using WR6026 as the test drug. Procedures 1 & 2 are described in Scheme I.

---

[a]Preparation 2 differs from 1 only in using a drug swelling solution containing some other drug other than WR6026

TABLE I

Chemicals Us in Liposome Preparation

Chemical	Supplier	Catalog No.
DL-α-phosphatidylcholine (DPPC)	Sigma	P-6138
	WRAIR	BJ 83128 Lot 01063
Cholesterol (CHOL)	Sigma	CH-S
	WRAIR	WR 13106AG
Vitamin E(DL-α-tocopherol)	Sigma	T-3251
Chloroform, Reagent Grade	Mallinkrodt	SP 4440-8
WR6026·2HCl	WRAIR	BH 64682 34 AM 87-2
Dicetylphosphate (DCP)	Sigma	D-2631
Stearylamine (SA)	Sigma	S-6755

SCHEME I

(Preparations 1 & 2)

DPPC - 200 micromoles (150 mg)
CHOL - 150 micromoles (58 mg)

Sufficient to make 10 x 2 ml aliquots

1. DPPC + CHOL dissolved in 15 ml of chloroform.
↓
2. Chloroform solution evaporated on a Rotavapor-R/A rotary evaporator at 30°C in a 100 ml round bottom flask.
↓
3. Further drying of lipids in a vacuum desiccator for 1¼ hours.
↓
4. Lipids "swollen" with 20 ml distilled water and 10-20 acid-washed glass beads (2-3 mm) by vigorous vortex mixing.
↓
5. Aliquots (2 ml) transferred to washed 10 ml lyophilization vails.
↓
6. Aliquots lyophilized for 24 hours with 24 hours further drying in a vacuum desiccator. All vials were finally stoppered and stored at 4°C.
↓
7. Lyophilized material "swollen" with 5 ml of a 1.04 mg/ml solution of WR6026·2HCl for each vial. "Swelling" and dispersion was accomplished by vigorous mixing (10 min.) on a vortex mixer.
↓
8. Dispersions were evaluated for degree of drug entrapment by ultrafiltration, centrifugation, dialysis and direct analysis.

Preparations 3, 4 and [illegible]

These procedures differed from those for Preparations 1 and 2 primarily in the method of incorportion of WR6026·2HCl (or other drug) at the first stage in the process shown in Scheme II. The previous procedures used the drug solution as a "swelling" solution in one of the latter steps, while for these preparations the drug becomes intimately mixed with the lipid by being dissolved with the lipid in the first organic solution.

Each of these preparations (i.e., 3, 4 or 5) differed only in whether the samples were lyophilized and how soon they were "swollen" after lyophilization.

Preparations 6 and 7

These two liposomal preparations were made by the same procedure as Preparations 3, 4, and 5 except that a total of 3 micromoles (1.29 mg) of vitamin E (DL-α-tocopherol) was incorporated in the original lipid-drug chloroform solution. This amounted to 0.2 micromoles of vitamin E per final 2 ml aliquot or sample. Preparation 7 was divided into two groups of 5 vials each. Group 7A was reconstituted just prior to entrapment evaluation and Group 7B was reconstituted at the end of the lyophilization process and stored in a refrigerator as a dispersion.

SCHEME II

(Preparations 3, 4 & 5)

DPPC - 300 micromoles (225 mg)
CHOL - 225 micromoles (87 mg)
WR6026·2HCl - 185 micromoles (77 mg)

Sufficient to make 15 x 2 ml aliquots

1. DPPC, CHOL and WR6026·2HCl dissolved in 20-25ml of chloroform.

2. Chloroform solution evaporated on a Rotavapor-R/A rotary evaporator at 30-35°C in a 100 ml round bottom flask.

↓

3. Further drying of the lipid-drug mixture in a vacuum desiccator for 2 hours.

↓

4. Lipid-drug mixture "swollen" in 30 ml of distilled water with 10-20 acid-washed glass beads (2-3 mm) by vigorous vortex mixing.

↓

5. Aliquots (2 ml) transferred to 15 washed 10 ml lyophilization vials.

10 vials

6. Aliquots lyophilized for 24 hours with 24 hours drying in a vacuum desiccator. All vials were finally stoppered and stored at 4°C.

5 vials

7. Aliquots flushed with nitrogen and stoppered. All vials were stored at 4°C. (Preparation 5)

5 vials

8. Lipid-drug mixture "swollen" in 2 ml distilled water with vigorous mixing (2 min.) on a vortex mixer at the time of entrapment evaluation. Vials were stored at 4°C in the lyophilized state. (Preparation 3)

5 vials

9. Lipid-drug mixture "swollen" in 2 ml of distilled water with vigorous mixing (2 min.), flushed with nitrogen and stoppered. Vials were stored at 4°C until entrapment evaluation. (Preparation 4)

10. Dispersions were evaluated for degree of drug entrapment by ultrafiltration, centrifugation, dialysis and direct analysis.

Modified Preparation Techniques

The previously described liposomes preparation techniques were modified as more experience was gained with these methods to improve the efficiency of preparation and to eliminate steps thought to decrease entrapment efficiency. These modifications included:

1. elimination of lyophilization of washed liposmes
2. increasing the temperature at which swelling of the liposmes was conducted
3. use of different agitation conditions for swelling lipid (i.e. gentle shaking in a shaker bath and ultrasonification)
4. extrusion of the liposomes through Nucleopore filters of various pore size to narrow and reduce liposome size

These modifications will be described more fully when they are employed.

METHODS FOR EVALUATING LIPOSOME ENTRAPMENT

Dialysis

A liposome dispersion (2 ml) containing entrapped and unentrapped drug was pipetted into a dialysis sac (molecular weight cutoff-12-14,00 or 50,000) and sealed with plastic closures. The dialysis sac was shaken for one hour in 150-200 ml of normal saline (N.S.) diluted appropriately to have the same tonicity as the drug concentration. This procedure was carried out 3-5 times with the dialyzate collected and pooled. The pH of the pooled dialyzate was adjusted to pH 2 with HCl and the solution assayed by measuring

the UV absorbance at 262 nm. The percentage entrapment was calculated by subtacting the amount recovered in the dialyzate from that originally put into the liposome system and dividing the difference by the amount of drug originally put into the system.

Centrifugation

The liposome dispersion (2 ml) containing entrapped and unentrapped drug was pipetted into a centrifuge tube and centrifuged in a swinging bucket centrifuge (International Centrifuge) at 2000 rpm for 15-30 minutes. The supernatant solution was collected and the liposomes redispersed in 25-35 ml of normal saline diluted appropriately to have the same tonicity as the drug concentration. This procedure was repeated 3-5 times with the supernatant collected each time and pooled. The pH of the pooled supernatant was adjusted to pH 2 with HCl and the solution assayed by measuring the UV absorbance at 262 nm. The percentage entrapment was calculated by subtracting the amount recovered in the supernatant from the amount of drug originally put into the liposome system and dividing the difference by the amount of drug originally put into the system.

Direct Analysis

The liposomes with entrapped drug remaining after complete dialysis or the final centrifugation were dissolved in isopropanol. The isopropanol solution was assayed by measuring its UV absorbance at 264 nm. The percentage entrapped was calculated by dividing the drug content of the

isopropanol solution by the amount of drug originally put into the system.

Ultrafiltration

An aliquot (0.5-2 ml) of the liposome dispersion containing entrapped and unentrapped was pipetted into an Amicon Centriflo cone and centrifuged at high speed which forced ultrafiltrate through the membrane. The ultrafiltrate was then assayed by measuring its UV absorbance at 262 nm after adjusting to pH 2 with HCl.

This procedure was found to be a measure of direct binding of drug to the liposomes but not a reliable estimate of entrapment.

UV ASSAY OF WR6026·2HCl IN VARIOUS MEDIA

In preparation for liposome entrapment studies with WR6026·2HCl, UV spectra and standard curves for WR6026·2HCl were obtained in various media over the range from 200 nm to 450 nm. All spectra were obtained using 1 cm pathlength quartz cuvettes with a Pye-Unicam 8-100 UV-Visible Recording Spectrophotometer. These spectra and Beer's law plots were reported earlier (2).

Table II contains the molar absorptivities of WR6026·2HCl in various media as well as the wavelength maximum. As can be readily seen by comparing the molar absorptivities in Table II, the spectral characteristics of WR6026·2HCl are quite pH and solvent dependent. The spectra in these solvents are shown at various concentrations in Figures 1-5.

In water, the spectral maxima shift slightly to higher wavelength with increasing concentration because the solution pH drops and the spectrum becomes closer to that found at pH 2 (0.01 N HCl). Between pH 2 and pH 6, WR6026·2HCl loses its first proton ($pK_{a1} \sim 4$) and thus the spectrum is a composite that of the doubly protonated molecule at pH 2 and that of the singly protonated molecule at pH 6. Spectra were not obtained at higher pH because the solubility of WR6026 drops dramatically as the molecule passes through its second pK_a. Based upon these spectral studies it was deemed necessary to adjust the pH of any solution of WR6026 to pH 2 or pH 6 to obtain reproducible absorptivities and spectra. In water or normal saline, the solution pH is dependent upon the drug concentration, CO_2 concentration and concentration of other solution additives to the extent that it may vary from pH 3.5-5.5 over which range the spectrum changes significantly. Therefore, all liposome entrapment assays were conducted by adjusting the final pH of aqueous dialyzates, washings, etc. to pH 2 before UV analysis.

For direct assay of WR6026 content in the liposomes after dialysis or repeated washing and centrifugation, ispropanol was found to be a satisfactory solvent. All liposome components had sufficient isopropanol solubility, including WR6026·2HCl, to make it a useful solvent to dissolve all the liposome contents for direct assay of drug content. Also, isopropanol has a low UV cutoff (205 nm) which makes it better for UV analysis than chloroform or

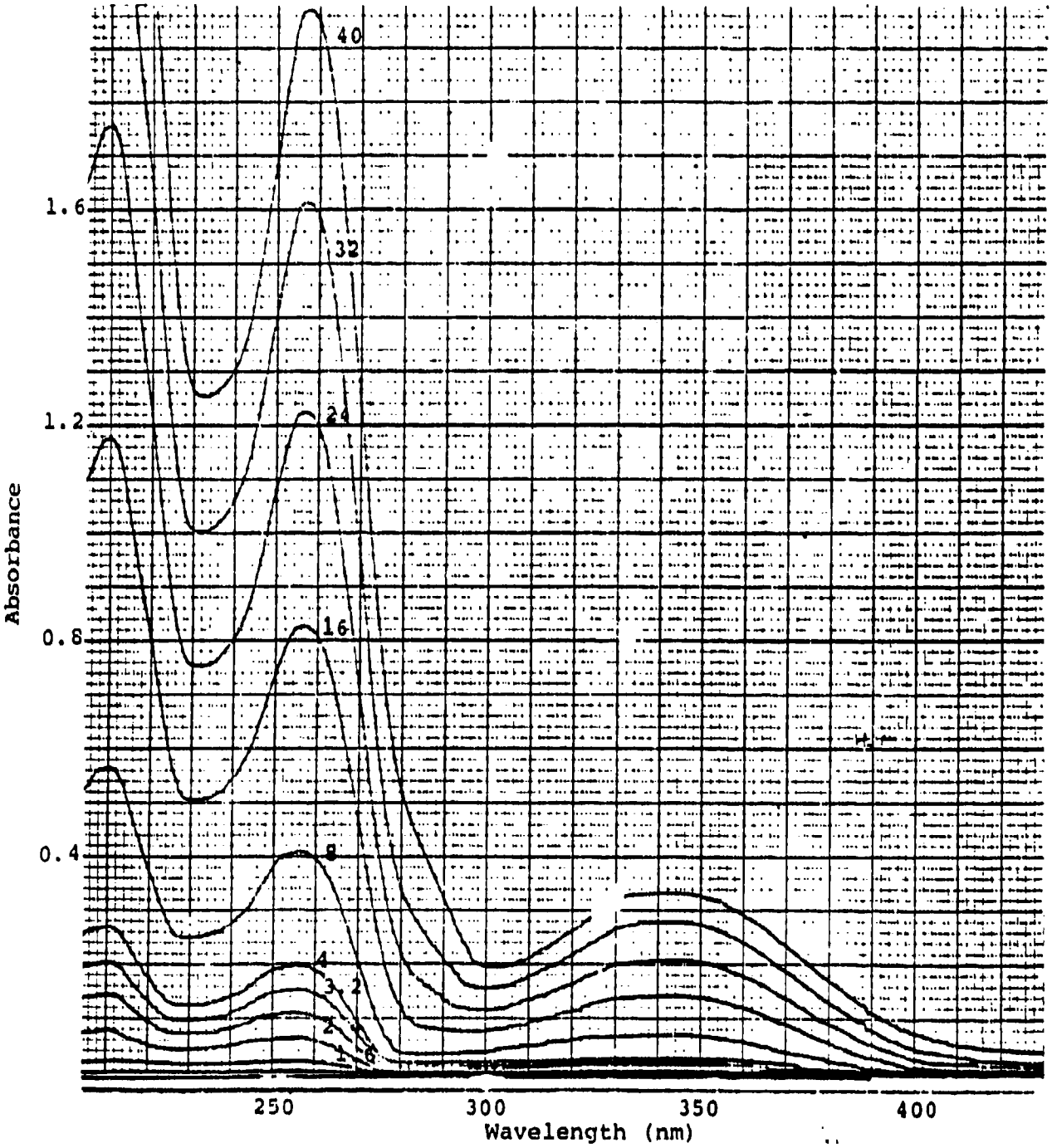

Figure 1. UV Spectrum of WR6026·2HCl in H_2O (concentrations are micrograms/ml)

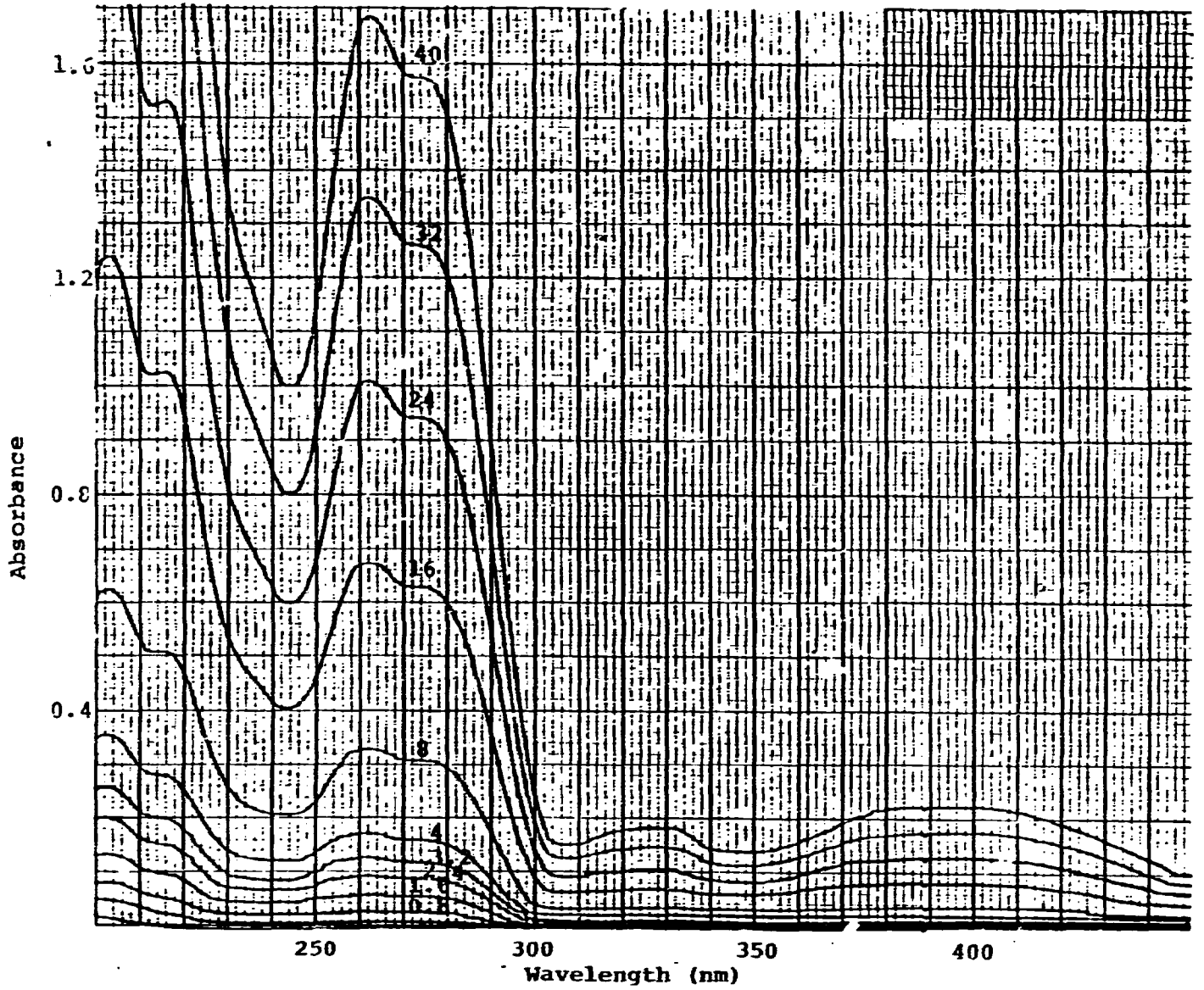

Figure 2. UV Spectrum of WR6026·2HCl in 0.01 N HCl (pH 2) (concentrations are micrograms/ml)

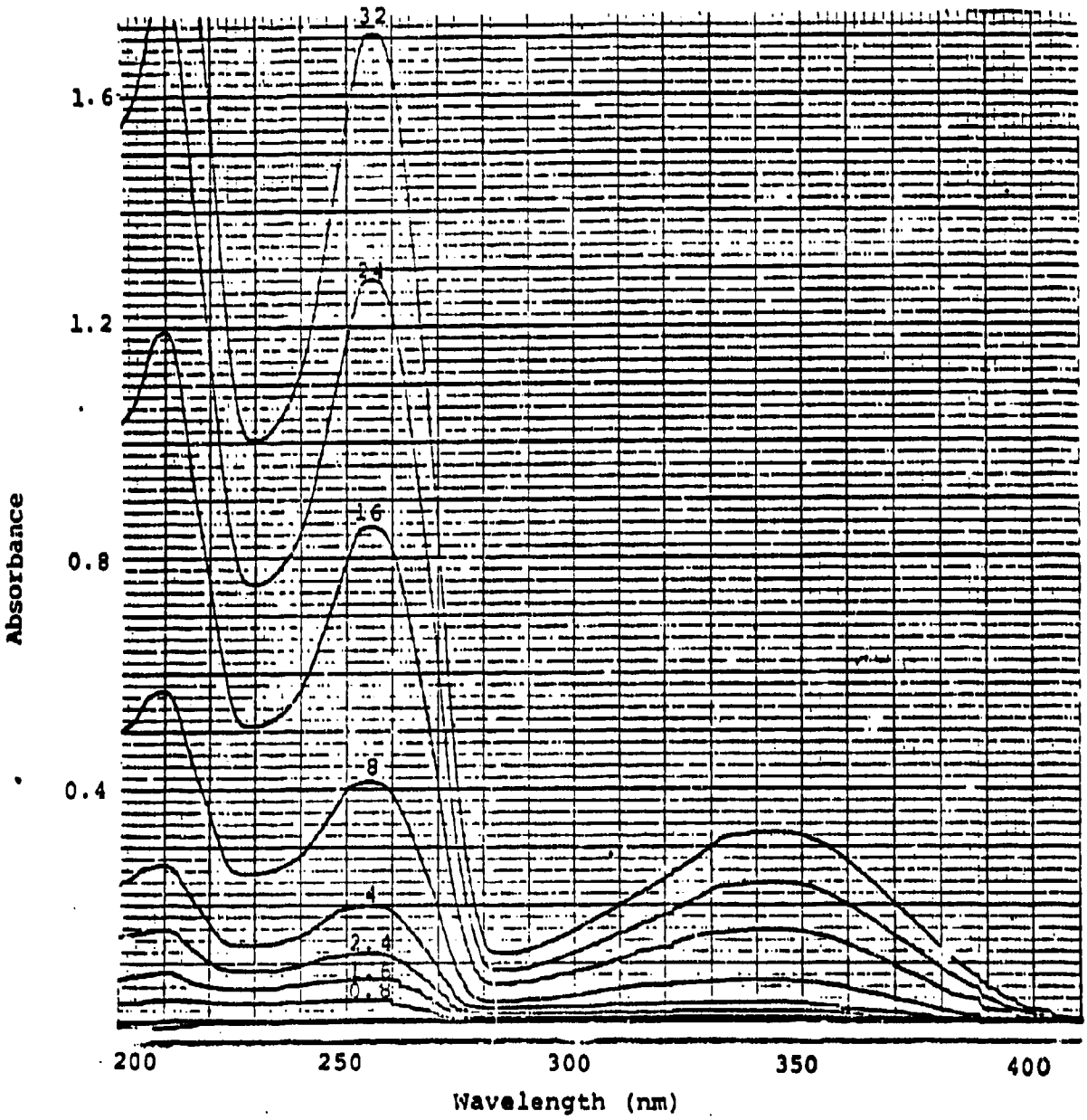

Figure 3. UV Spectrum of WR6026·2HCl in 1/15 M Phosphate Buffer (pH 6) (concentrations are micrograms/ml)

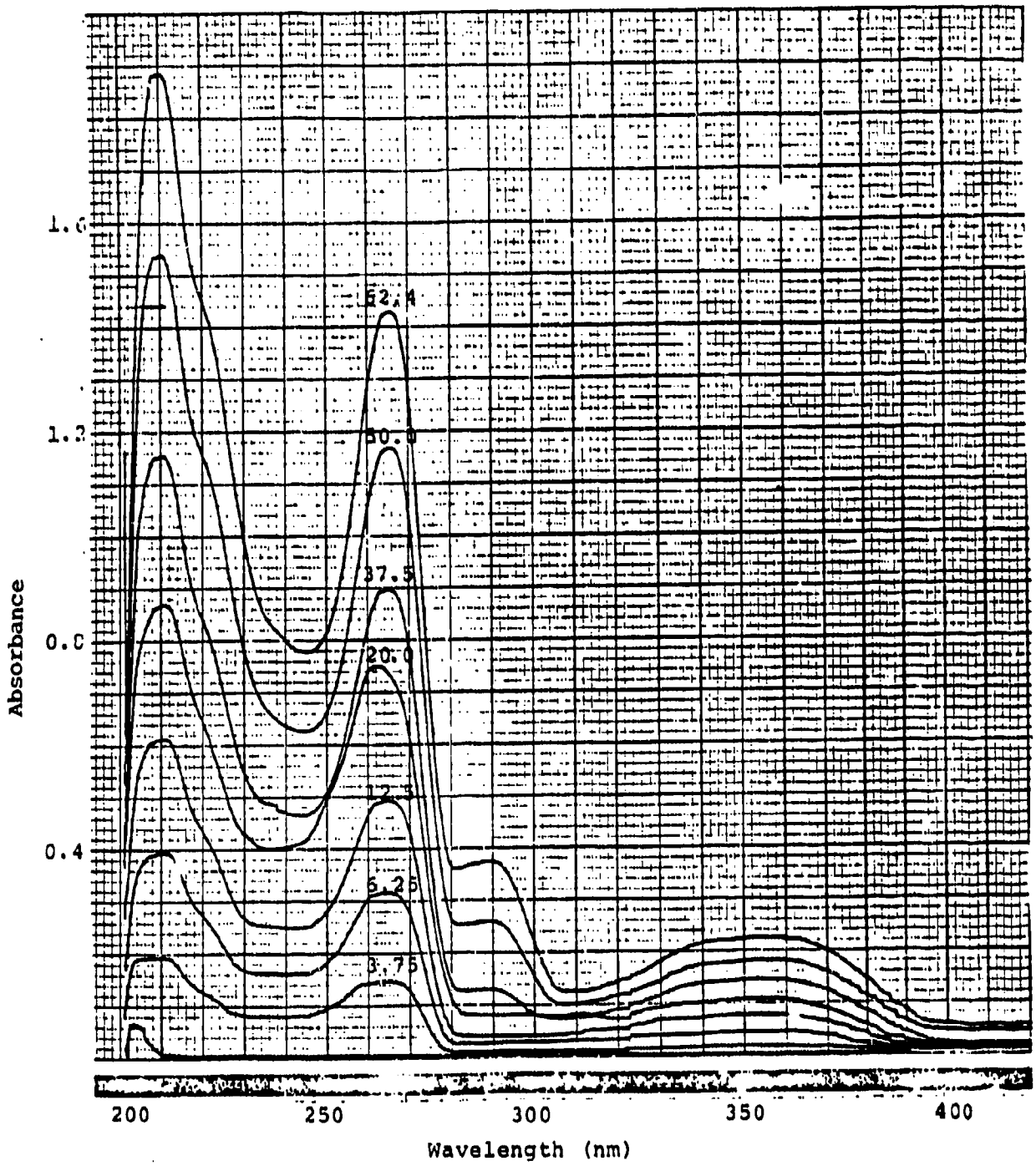

Figure 4. UV Spectrum of WR6026·2HCl in Isopropanol (concentrations are micromolar)

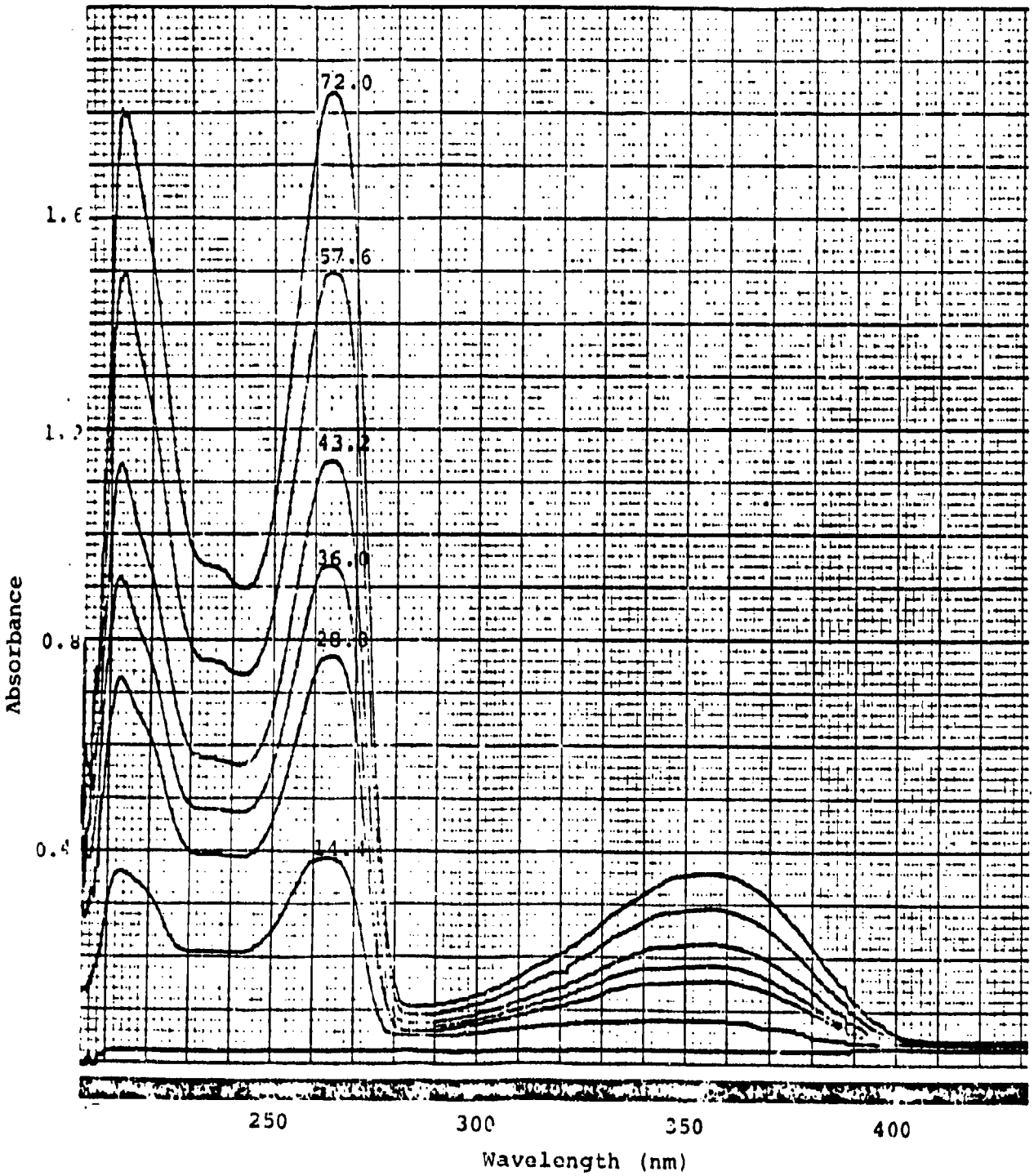

Figure 5. UV Spectrum of WR6026·2HCl in Isopropanol containing 3.2% H_2O, 0.1856 mg/ml cholesterol and 0.48 mg/ml lecithin (concentrations are micromolar)

methylene chloride which have much higher cutoff wavelengths, even though these solvents are also satisfactory for dissolving the liposomes.

Table II. Molar Absorptivities of WR6026·2HCl in Various Media

Medium	Wavelength (nm)	Molar Absorptivity
H_2O	258	20,922
Normal Saline	258	19,913
0.01 N HCl (pH 2)	262	17,548
1/15 M Phosphate Buffer (pH 6)	256	22,260
Isopropyl Alcohol	264	23,240
Isopropyl Alcohol (containing 3.2% H_2O 0.0856 mg/ml cholesterol and 0.48 mg/ml lecithin)	264	25,489

TONICITY OF WR6026·2HCl AND PRIMAQUINE PHOSPHATE (WR2975) SOLUTIONS

Since liposomes act as "osmometers" by swelling or shrinking in response to the tonicity of solutions in which they dispersed, it is important to know the tonicity of drug solutions entrapped in liposomes. Serum osmolality is nominally taken to be 300 mOsm/kg. USP XX requires labelling the osmolarity of Pharmacopeial solutions that provide intravenous replenishment of fluid, nutrients or electrolytes, as well as of the osmotic diuretic, Mannitol Injection. A 0.9% sodium chloride solution which is iso-osmotic with serum has been experimentally found to have an osmolality of 288 mOsm/kg (3).

With this in mind the osmolality of WR6026·2HCl and primaquine phosphate (WR2975) solutions was determined by freezing point depression to obtain the concentration of each drug which is isotonic with serum. The actual determinations were performed by the Clinical Medicine Laboratories of the University of Iowa Hospitals and Clinics with an Advanced Instruments Model 3CII Cryomatic Osmometer. Table III shows the osmolalities obtained for various concentrations of each drug. From this data it was found that a 250 mM solution of WR6026·2HCl and a 167.8 mM solution of primaquine phosphate were isotonic with serum. Both values represent significant deviations from ideal behavior for electrolytes which generate three ions in solutions. Nominally such salts should have isotonic concentrations around 100 mM. Values larger than this indicate non-ideal solution behavior most likely due to self-association in solution. The value for primaquine is over 50% higher than the ideal value of 100 mM which is not too unusual since a number of cationic drugs show such deviations from ideality (4). The value for WR6026 approaches the iso-osmotic concentration for a non-electrolyte which is quite unusual. WR6026 appears to self-associate to a high degree as an explanation for iso-osmotic concentration of 250 mM when an ideal non-electrolyte would be iso-osmotic at 288 mM. The self-association behavior of WR6026 warrants further investigation because it may account for its high aqueous solubility and may affect its stability in solution.

For liposome entrapment studies, the isotonic concentration of WR6026·HCl was taken to be 250 mM (104 mg/ml) and that of primaquine phosphate was taken to be 167.8 mM (76.4 mg/ml).

Table III. Osmolality of WR6026·2HCl and Primaquine Phosphate Solutions

Concentration (mM)	Osmolality (mOsm/kg)	
	WR6026·2HCl	Primaquine Phosphate
100	169	130
120	152,157	209,205
150	192	262
167.8	-	287
200	238	-
233	-	485
250	285	-

LIPOSOME ENTRAPMENT EFFICIENCY

Stage One

As was mentioned in the introduction, the first entrapment studies of WR6026·2HCl were conducted at a low swelling solution concentration of approximately 1 mg/ml. Tables IV-VI give the entrapment efficiencies for each preparation technique described earlier.

TABLE IV

Entrapment Results for Preparatons 1 & 2

Evaluation Method	% Entrapped
Ultrafiltration	15-17
Centrifugation	0
Dialysis	5

TABLE V

Entrapment Results for Preparations 3, 4 & 5

Evaluation Method		% Entrapped Preparation 3	Preparation 4	Preparation 5
Ultrafiltration		34.6	29.4	14.3
Centrifugation		13.75	6.8	15.5
Dialysis	53 hrs	23.0	20.45	16.0
	131 hrs	14.0	14.6	-
	175 hrs	10.15	11.5	-

TABLE VI

Entrapment Results for Preparations 6 & 7

Evaluation Method		% Entrapped Preparation 6	Preparation 7A	Preparation 7B
Ultrafiltration		20.9	28.7	24.5
Dialysis	24 hrs	21.9	20.5	24.0
	48 hrs	17.0	18.3	20.3
	96 hrs	10.6	13.6	16.3
	120 hrs	8.4	12.1	15.5
	168 hrs	7.8	11.0	13.0

As can be readily seen each evaluation procedure give apparently different entrapment results. We found that the ultrafiltration technique consistently give entrapment values that were too high, probably due to binding of WR6026 to the ultrafiltration membrane. Thus ultrafiltration was not used in further entrapment studies.

The centrifugation and dialysis results also differ signficiantly. Since these early studies did not involve direct assay of WR6026 in the washed liposomes, it is unclear which are the more reliable values. Later studies with 1 mg/ml swelling solutions did not give such high entrapment efficiencies. The main point in reviewing these early results is to show that upon prolonged dialysis the liposomes will "leak" their contents (Tables V and VI) and thus it would be advisable to store any swollen liposome dispersions in the unwashed state until administration. The dispersion could be easily "washed" by repeated (3x) centrifugation and resuspension in normal saline or other suitable vehicle immediately before use.

Stage Two

Later entrapment studies, described in the introduction as our second stage are summarized in Table VII-IX. For each concentration of WR6026, entrapment was evaluated by dialysis, centrifugation and direct analysis as described earlier. The dialyzing or washing solutions were appropriate dilutions of normal saline to maintain the tonicity across the liposome membrane.

Table VII. Entrapment Efficiency for WR 6026 in Liposomes by Method 1[a,b]

Drug Conc. (mg/ml)	Sigma Lipids	
	Dialysis	Centrifugation
1 (N.S. x 1/100)[d]	5.09 (1.97)[c]	1.53 (1.52)
10 (N.S. x 1/10)	3.54 (4.39)	1.29 (3.72)
25 (N.S. x 1/4)	7.51 (7.75)	0.72 (0.25)

[a]Method 1 employed flash evaporation of lipids in chloroform, swelling with drug solution and lyophilization of liposome dispersion. Entrapment assessed after reconstitution of lyophilized material.

[b]Values are % entrapment based on drug concentration in swelling solution.

[c]Values in parentheses are % entrapment assessed by direct analysis after dialysis or centrifugation.

[d]Dialyzing or washing solutions as appropriate dilutions of normal saline (N.S.).

Table VIII. Entrapment Efficiency for WR 6026 in Liposomes by Method 1 without Lyophilization[a,b]

Drug Conc. (mg/ml)	Sigma Lipids		WRAIR Lipids	
	Dialysis	Centrifugation	Dialysis	Centrifugation
1 (N.S. x 1/100)[d]	5.68 (4.42)[c]	1.53 (3.14)	3.90 (4.37)	3.30 (3.63)
10 (N.S. x 1/10)	4.79 (1.79)	1.53 (-)[e]	2.12 (2.33)	2.12 (-)[e]
25 (N.S. x 1/4)	3.66 (1.53)	2.24 (0.37)	3.19 (1.41)	1.76 (0.55)
52 (N.S. x 1/2)	6.26 (2.03)	6.81 (0.66)	5.99 (2.41)	6.89 (0.56)
104 (N.S.)	1.92 (3.89)	6.85 (1.64)	3.93 (2.39)	4.16 (1.60)

[a] Method 1 employs flash evaporation of lipids in chloroform and swelling with drug solution. Entrapment assessed after swelling.

[b] Values are % entrapment based on drug concentration after swelling.

[c] Values in parentheses are % entrapment assessed by direct analysis after dialysis or centrifugation.

[d] Dialyzing or washing solutions as appropriate dilutions of normal saline (N.S.).

[e] Procedure not done.

Table IX. Entrapment Efficiency for WR 6026 in Liposomes by Methods 3, 4 & 5 without Lyophilization[a,b]

Drug Conc. (mg/ml)	Sigma Lipids		WRAIR Lipids	
	Dialysis	Centrifugation	Dialysis	Centrifugation
1 (N.S. x 1/100)[d]	12.5 (11.65)[c]	-[e] (-)	13.24 (16.31)	-[e] (-)
10 (N.S. x 1/10)	6.27 (6.33)	3.20 (3.38)	9.24 (8.30)	3.76 (4.22)
25 (N.S. x 1/4)	4.49 (7.24)	4.98 (3.32)	7.00 (6.13)	4.95 (2.65)
50 (N.S. x 1/2)	6.27 (2.04)	6.26 (1.77)	5.68 (2.03)	6.25 (1.77)
104 (N.S.)	10.45 (3.31)	8.74 (1.36)	9.87 (2.26)	11.57 (1.33)

[a]Methods 3, 4 & 5 employ flash evaporation of lipids in chloroform with drug and swelling with distilled water. Entrapment assessed after swelling.

[b]Values are % entrapment based on drug concentration after swelling.

[c]Values in parentheses are % entrapment assessed by direct analysis after dialysis or centrifugation.

[d]Dialyzing or washing solutions as appropriate dilutions of normal saline (N.S.).

[e]Liposome dispersion could not be completely centrifuged. Dispersion remained turbid after centrifugation.

It is clear from these studies, as well, that entrapment appears to vary with drug concentration and method of evaluation. In general, centrifugation gives a lower % entrapped compared to dialysis. This could be due to liposome rupture during centrifugation, incomplete centrifugation of sub-micron liposomes which are not observable to the eye or possibly dialysis is not complete. It is difficult to determine which of these is the source of the difference between these two techniques. We feel that the dialysis is reasonably complete after 3-5 equilibrations with fresh dialyzing solution over a total of 5 hours. Dr. Alving has stated that liposomes are fairly resistant to rupture during centrifugation. Therefore, the problem may be in not completely centrifuging the liposomes, especially those below one micron which would not be observable to the eye.

One important aspect with both of these methods is that they involve subtracting two large values from one another (the original amount of drug put in minus the amount removed by dialysis or centrifugation). Any small variations (1-5%) in either of these values will be greatly magnified in variation of the difference. For example, when the liposomes are swollen by adding a specific volume of swelling solution (either distilled water or drug solution depending upon the method) if the final liposome dispersion volume expands or contracts a few percent, then the actual drug content of a particular volume aliquot varies a few percent also from what was calculated for the system initially. This may not

seem significant but when the amount dialyzed or decanted off is subtracted from the amount originally put into a dispersion aliquot, if one of these values varies by a few percent the difference may vary 25-50% since the two values are close to each other.

Thus, direct analysis which is not subject to such large variation, is probably the more reliable entrapment assessment method since it does not involve taking the difference between two values. In most cases the direct method gives lower % entrapment than the indirect methods (dialysis and centrifugation). Also significant variability is observed between direct analysis after dialysis versus centrifugation. The source of this variability is not clear but at least it can be stated that approximately 1-3% of WR6026 is entrapped at 104 mg/ml with these preparation techniques, which would be the optimal swelling concentration to use since it is the isotonic concentration of WR6026.

It should also be noted that the dispersions take on a different degree of "graininess" as the swelling concentration is increased. At high drug concentrations, the liposome dispersion looks coarse indicating large liposomes or large aggregates are present. Under the microscope the dispersion looks like aggregates of liposomes or lipid that did not properly hydrate to spherical liposomes. Figures 7-14 are photomicrographs of liposomes obtained after swelling with various WR6026·2HCl solutions. With no drug present (Figure 7) or at low concentration (1 mg/ml, Figures 8 and 9) the liposomes

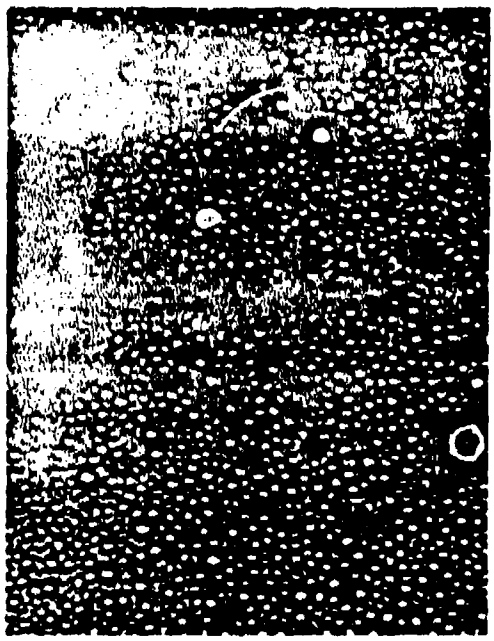

Figure 6. Photomicrograph (420x) of liposomes swollen in water

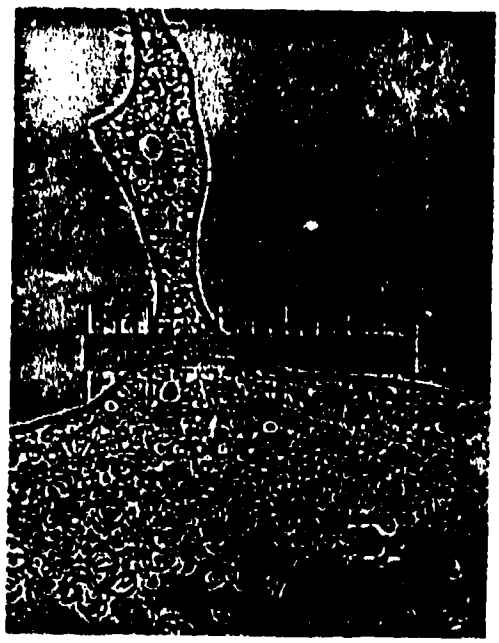

Figure 7. Photomicrograph (100x) of liposomes swollen in 1 mg/ml WR6026

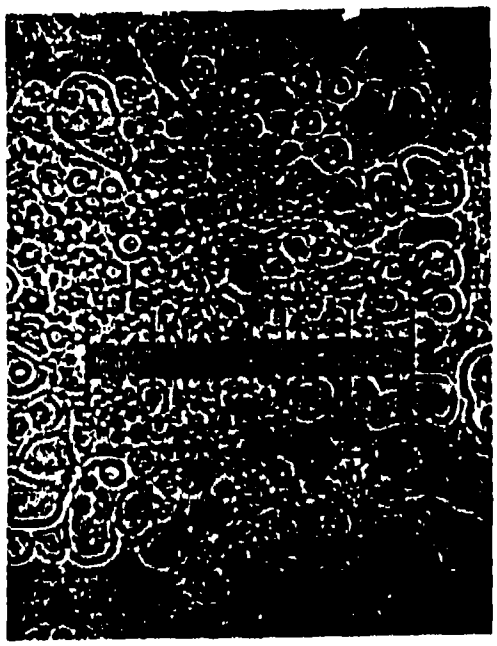

Figure 8. Photomicrograph (420x) of liposomes swollen in 1 mg/ml WR6026

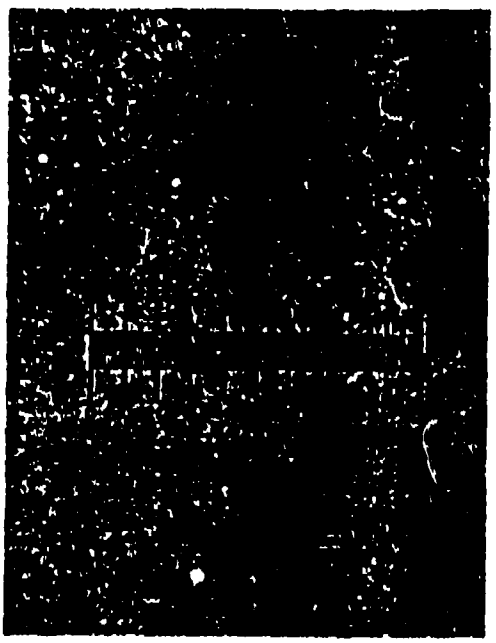

Figure 9. Photomicrograph (100x) of liposomes swollen in 10 mg/ml WR6026

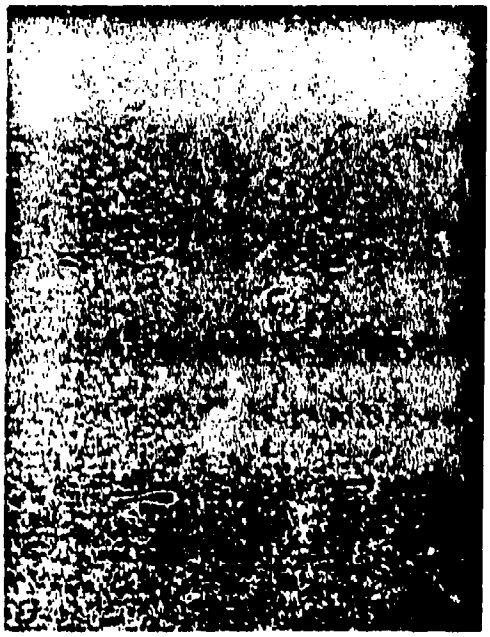

Figure 10. Photomicrograph (420x) of liposomes swollen in 10 mg/ml WR6026

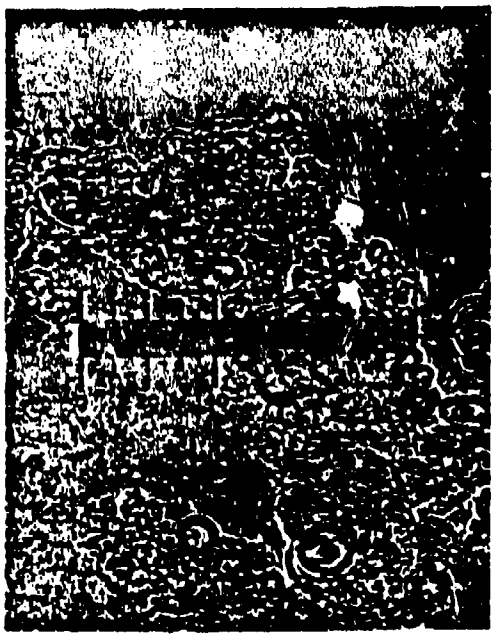

Figure 11. Photomicrograph (420x) of liposomes swollen in 25 mg/ml WR6026

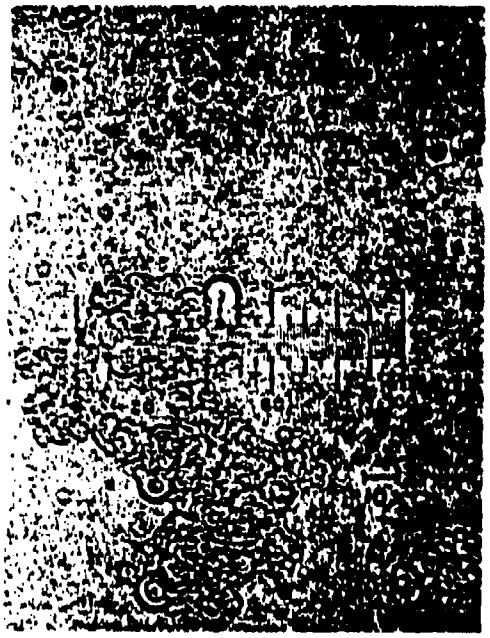

Figure 12. Photomicrograph (420x) of liposomes swollen in 50 mg/ml WR6026

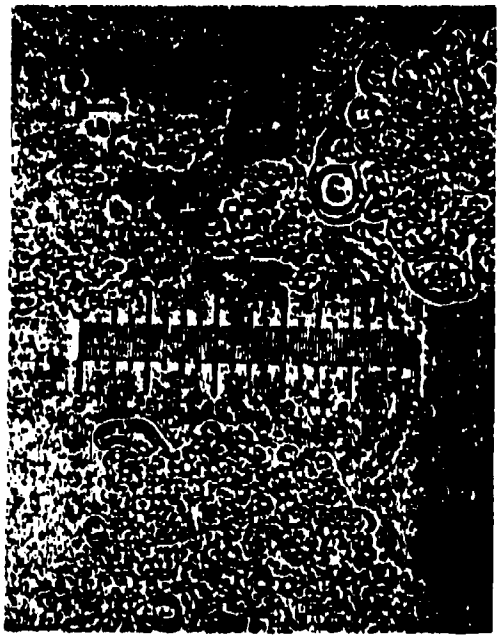

Figure 13. Photomicrograph of liposomes swollen in 104 mg/ml WR6026

appear as individual entities. Macroscopically these dispersions appear to be fine suspensions which are more difficult to centrifuge.

At 10 mg/ml (Figure 10 and 11) and above (Figure 12, 13 and 14), the liposomes appear aggregated as mentioned above. It appears that WR6026 is causing the liposomes to aggregate or interfering with liposome formation at higher concentrations.

It is likely that WR6026·2HCl interacts directly with the lipid components of the liposome. Even if the interaction is not particularily strong, the high concentration of WR6026 used in the swelling solution could push any equilibrium reaction involving complexation with cholesterol or lecithin far toward formation. On a mole basis at 104 mg/ml (250 mM), the ratio of WR6026 to lipid is approximately 15:1. In other words, the lipid is called upon to entrap drug solution that contains a fifteen-fold higher concentration of drug. At 1 mg/ml, the molar ratio of lipid to WR6026 favors the lipid by about 7:1. Thus it is not surprising that reasonably well formed liposomes are only generated at low drug concentration where there is much more lipid than drug in the system.

Stage Three

After our initial experience with these liposomal systems, modifications in the preparation methods were employed to improve entrapment efficiency. A one day discussion of our initial results was held with Dr. Tony Hunt of the University of California Medical Center in San Francisco and

Dr. Larry Fleckenstein of WRAIR in April. Dr. Hunt made a number of recommendation that we have implemented to determine whether this liposome system could be optimized further.

Among his suggestions were to employ more gentle shaking (with no beads) at elevated temperature (~40°C) to promote better hydration of the lipid. The elevated temperature would insure that the lecithin-cholesterol bilayer was above its transition temperature (T_c) so that it would have significant flexibility for forming spheres rather than being stiff and/or brittle.

The lyophilization step was eliminated as a time consuming process (1-2 day/run) that is not enhancing entrapment efficiency. Lyophilization can be investigated again as a preservation method for the lipid once other problems have been dealt with.

Dr. Hunt has seen that pushing liposomes through thin membrane filters (i.e., Nucleopore) can narrow their size distributions, reduce their average size and increase entrapment (5). The liposomes can actually "squeeze" through the pores of the filter with the excess lipid on large liposomes being sheared off to from more liposomes. Thus, the larger liposomes are not filtered out but rather are broken down during the filtration process to form many smaller liposomes which can entrap more swelling solution as they form.

We also decided to add a charge to the liposomes by incorporating dicetylphosphate (DCP) or stearylamine (SA) to the lipid formulations. In the typical lipid formulation

the molar ratio of lecithin to cholesterol is 5:4. To produce either a negative charge (using (DCP) or a positive charge (using SA), one part of either of these two materials was added giving a molar ratio of 5:4:1 (DPPC:Cholesterol:SA or DCP).

Our initial studies with gentle agitation in a thermostated (40°C) shaker bath without glass beads or vortex mixing were at a 104 mg/ml concentration of WR6026. After 21 hours of shaking, some lipid still remained on the flask wall. If normal saline (N.S.) was used under the same agitation conditions to swell the lipid it took less than five minutes for the lipid to swell and disperse. This observation lends credence to the suggestion that WR6026 interferes in some fashion with the normal hydration of the lipid. The dispersions thus produced with WR6026 looked coarse and gave only an average entrapment for four samples of 0.87%.[1] After extruding (filtering) the dispersion three times through a 12 micron Nucleopore filter the entrapment for four samples still averaged only 0.82%.

This entrapment process was repeated with intermittant vortex mixing in addition to the gentle shaking at 40°C to remove the lipid from the flask wall faster. By this procedure, swelling was completed in one hour but the entrapment was only 1.09% of a 104 mg/ml solution.

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[1]All entrapments will now be based upon direct assay of washed liposomes in isopropanol after centrifugation and washing in appropriately diluted normal saline.

Lower swelling solution concentrations were used (i.e. 5 and 10 mg/ml) to determine if higher entrapments could still be achieved with this gentle dispersion method. At 5 mg/ml, the lipid dispersed in about 10 minutes and the entrapment averaged 14.7% for two samples (in this case the two sample differed significantly; one being 12% and the other being 17.4%). At 10 mg/ml, the lipid dispersed in about 30 minutes and gave an average entrapment of 5.2% (6.1% and 4.3%). Extruding (filtering) these dispersions three times through an 8 micron Nucleopore filter, did not change the entrapment significantly. The 5 mg/ml dispersion averaged 14.4% entrapped (12.0 and 16.9%) and the 10 mg/ml dispersion averaged 5.7% entrapped (4.7 and 6.6%).

Even though the extrusion process did not enhance entrapment, it is significant that essentially all of the dispersion passed through the filter with out apparent loss of drug or lipid. This, it is conceivable that these dispersions could be microfiltered as a cold sterilization technique without great concern about loss of drug. Also, as noted above, the lipid dispersed more rapidly with the lower drug concentrations (i.e., 5 and 10 mg/ml) than with the high concentration (i.e., 104 mg/ml) indicating again that WR6026 probably interacts with the lipid material or interferes with its hydration.

When dicetylphosphate (DCP) or stearylamine (SA) were incorporated into the liposome lipid at 1 mole/9 moles lipid (5 moles DPPC + 4 moles Chol.), the entrapment of a 5 mg/ml

changed significantly for the worse. Even though the lipid dispersed within 15-45 minutes, the DCP-containing liposomes entrapped only 4.9% and the SA-containing lipo mes entrapped only 1.9% of the swelling solution. Extrusion ree times through a 1 micron Nucleopore filter only increased entrapment to 5.5% for the DCP liposomes while the SA liposomes were very small and could not be centrifuged to be washed after extrusion. It is not surprising that the positively charged liposomes containing SA had lower entrapment characteristics since WR6026 is positively charged and could be repelled from the liposomes as they formed. It is stran,e that the negative DCP liposomes showed poorer entrapment since WR6026 should be attracted into these liposomes.

The same DCP- and SA-containing liposome systems were used with a 104 mg/ml WR6026 swelling solution. In this case the negative DCP liposomes entrapped an average of 5.8% (6.7 and 5.0%) while the positive SA liposomes entrapped an average of 1.1% (1.5 and 0.8%). An additional change in the process introduced at this point was the employment of ultrasonic radiation to aid in dispersing the lipid. After 1-2 days of gentle agitation both liposome dispersion systems were subjected five times to 30 second exposures of ultrasonic radiation in a small ultrasonic bath. The 5.8% entrapament with the negative DCP liposomes was the highest consistent value obtained up to that time for the high concentration swelling solution of WR6026.

The entrapment study in the negative DCP system was repeated and compared to a neutral liposome system since it was possible that the entrapment enhancement could have been due to the use of ultrasonics rather than DCP. The repeat study gave an average entrapment of 4.9% (4.8 and 5.1%) for the negative DCP liposmes while the neutral liposomes gave an average of 1.0% entrapment (1.5 and 0.6%).

A similar comparison between negative and neutral liposomes at a 10 mg/ml swelling concentration using ultrasonic radiation was conducted. In this case twenty 5 second bursts of ultrasonic radiation were used after 30 minutes of gentle shaking at 40°C. The negative liposomes gave an average entrapment of 3.9% (3.7 and 4.0%) while the neutral liposomes gave 2.5% (2.3 and 2.8%). It appears that neither 5 mg/ml nor 10 mg/ml swelling solutions are better entrapped in negative liposomes and the ultrasonic radiation does not promote better entrapment at these levels.

The Gibco liposome kits described in our previous report (6) were evaluated to determine if they offered any advantages over our standard liposome formulation. The Gibco kit contains purified egg lecithin, cholesterol and dicetylphosphate in the mole ratio of 7:1:2. A swelling solution concentration of 104 mg/ml was employed. The negative Gibco liposome kit gave an average of 3.00% (both samples were 3.0%) entrapped. If dicetylphosphate was not added to the lipid, producing a neutral liposome, the entrapment was only 1.1% (1.0 and 1.2%). Thus, the Gibco lipid

combination does not offer any significant advantage in entrapment efficiency compared to our other liposome formulations.

When the negatve Gibco liposome kit was used with a 5 mg/ml swelling solution the entrapment efficiency went up to 14.8% (15.6 and 14.0%) but similar results had been previously obtained with 5 mg/ml with our standard neutral liposome formulation.

Since there had previously been shown to be a difference in degree of entrapment when the drug was added to the organic lipid solution before flash evaporation (Methods 3, 4 and 5), it was decided that a similar comparison should be made again with our modified preparation tehcniques. Thus neutral and negative lipid formulations were dissolved along with WR6026 in chloroform and the solution flash evaporated. WR6026 was added in amounts to give concentrations of 104 mg/ml and 5 mg/ml after swelling in water. Gentle agitation at 40°C was again used but no ultrasonification was employed. At 104 mg/ml, negative liposomes gave an average of 2.8% entrapment (1.9 and 3.6%) while neutral liposomes gave 2.5% entrapment (2.5 and 2.4%). No significant increase in entrapment was observed in this case although the neutral liposomes exhibited less variation. At 5 mg/ml, the negative liposomes gave an average entrapment of 9.3% (9.0 and 9.5%), while the neutral liposomes gave 21.9% (20.4 and 23.4%). This high entrapment in neutral liposomes is the highest consistent value obtained at any drug concentration and

represents the same amount of drug being entrapped with some of our poorer liposome preparations at 104 mg/ml swelling concentrations. Thus it appears that the 5 mg/ml concentration under these preparation conditions would give as high an incorporation as the much higher concentrations, which would involve less loss of unentrapped drug.

We had considered using a lower swelling solution volume with a fixed lipid amount to determine whether entrapment could be increased by elevating the concentration of lipid. One potential problem was the increased viscosity of a more concentrated lipid or liposome dispersions which could decrease the efficiency of hydrating the lipid. We conducted two such studies at 104 mg/ml and 5 mg/ml with one-half the normal swelling solution volume. In these systems the drug was added in the swelling solution to the lipid. In a negative liposome formulation, dispersed by gentle shaking at 40°C for 10 hours and then ultrasonification (12 x 5 sec.), an average of 15.6% (16.2 and 15.0%) of the 104 mg/ml swelling concentration was entrapped. At 5 mg/ml, in a neutral liposome formulation swollen by gentle shaking at 40°C for 20 minutes without subsequent ultrasonification, the average entrapment was 23.8% (23.6 and 23.9%). Thus, in the case of the negative liposome formulation the reduction in swelling volume gave an enormous increase in entrapment which is around four-fold higher than the best obtained with a 104 mg/ml swelling concentration. On the other hand, no significant enhancement was seen at 5 mg/ml by the volume

reduction, probably because the maximal entrapment limit is at 20-25%. Such high entrapments must be viewed cautiously since they must represent significant binding to or incorporation of WR6026 into the lipid bilayer membrane of the liposome. Theoretically one could calculate by an engulfing mechanism alone, that only 3.5-7.0% entrapment would be expected at the best. We are investigating these values more closely to ensure that they are reproducible.

One futher approach was suggested for getting WR6026 into the liposomes-that of forcing it in by pH adjustment. Neutral liposomes were prepared by swelling the lipid in appropriately diluted normal saline, the drug solution was then added to centrifuged liposomes and they were dispersed in the drug solution. The pH in one case was adjusted to pH 6 and in a second case to pH 10 to determine whether the monoprotonated form of WR6026 (at pH 6) or the uncharged form (at pH 10) could pass through the liposome wall and enter the liposome by a permeation. At 5 mg/ml, the pH 6 system gave an average of 1.8% entrapped (both samples 1.8%) if the liposomes were equilibrated over 14 hours with gentle shaking at 40°C while if ultrasonification was used to promote permeation only 1.0% was entrapped (0.9 and 1.05%). At 5 mg/ml, the pH 10 system gave an average of much less than 1% entrapment no matter how the liposomes were equilibrated with the liposomes. The results at pH 10 are not surprising since the aqueous solubility of WR6026 drops to a very low value at high pH and it precipitates after pH adjustment.

At pH 6, the entrapment observed is probably due primarily to WR6026 bound to the liposome rather than to material which actually penetrated to the inside of the liposome. It is our feeling that adjustment of pH does not appear at this time to be a viable route for incorporation of WR6026 in liposomes.

LIPOSOME PREPARATIONS SUBMITTED FOR ELECTRON MICROSCOPIC ANALYSIS

The following liposome preparatons were submitted for electron microscopy analysis by Dr. Masamichi Alkawa of Case-Western Reserve University:

Preparation	Description
1	4 parts DPPC:3 parts Cholesterol (molar ratios). Swollen with normal saline (Contains no drug).
2	4 parts DPPC:3 parts Cholesterol:1 part Dicetylphosphate (molar ratios). Swollen with normal saline (Contains no drug).
3	4 parts DPPC:3 parts Cholesterol (molar ratios). Swollen in isotonic WR6026 (104 mg/ml). Unwashed aliquot.
3W	Liposomes from preparation 3 washed and centrifuged three times. Dispersed in normal saline.
4	4 parts DPPC:3 parts Cholesterol (molar ratios). Swollen in 10 mg/ml WR6026. Unwashed aliquot.
4W	Liposomes from preparation 4 washed and centrifuged three times. Dispersed in 0.09% saline solution.
5	4 parts DPPC:3 parts Cholesterol (molar ratios). Swollen in 5 mg/ml WR6026. Unwashed aliquot.
5W	Liposomes from preparation 5 washed and centrifuged three times. Dispersed in 0.045% saline.

6	4 parts DPPC:3 parts Cholesterol:1 part Dicetylphosphate (molar ratio). Swollen in isotonic WR6026 (104 mg/ml). Unwashed aliquot.
6W	Liposomes from preparation 6 washed and centrifuged three times. Dispersed in normal saline.

At the time of this report we are awaiting the electron microscopic analysis of these samples to determine the size, shape and other structural characteristics of the liposomes.

ENTRAPMENT STUDIES ON PRIMAQUINE PHOSPHATE (WR2975)

Since Dr. Alving had also conducted liposomal studies on primaquine phosphate (7), it was decided to look at its entrapment in our laboratory. In these studies our standard neutral lipid was used and primaquine was added to the original lipid solution before flash evaporation. In this case a chloroform/isopropanol solution was used to dissolve the lipids used because the primaquine was not soluble enough in pure chloroform. Other than this modification, the preparation procedure was the same as Methods 3, 4 and 5 described earlier without lyophilization. The amount of primaquine phosphate used was sufficient to give an isotonic concentration (76.4 mg/ml or 167.9 mM) upon swelling with water.

The entrapment was assessed by centrifuging and washing the liposomes 5 times with normal saline and then dissolving the washed liposomes in isopropanol. The UV absorbance of the dissolved liposmes was measured at 266 nm (ε = 26,019) to determine the quantity of primaquine entrapped. The

average entrapment for seven samples was 0.7% (samples ranged from 0.5% to 0.9%).

A comparative assay of entrapment was conducted on the washed liposomes by a colorimetric assay according to the method of Fiske and Subbarrow (8). This assay method was used because Alving (7) had used a phosphate assay to assess entrapment. Assaying the same seven samples of dissolved liposomes for phosphate gave an average of 0.12% entrapment which is substantially lower than that obtained by UV analysis. It is our feeling that the phosphate exchanges across the liposome wall with chloride during washing and is not a reliable assessment of entrapment. In Alving's case he mentions only one washing step which may retain more phosphate but also may retain more unentrapped drug. It would seem prudent to conduct any future liposome work with a more specific assay for primaquine (i.e., UV or fluorescent analysis).

We made no further attempts to optimize primaquine entrapment in the liposomes. If it is deemed worthwhile, we could attempt to maximize primaquine entrapment to serve as a second drug with which to compare liposome entrapped WR6026. In fact, based upon Alving's limited test results (7), primaquine may be almost as effective as WR6026 in liposomes although he notes that different batches gave widely different <u>in vivo</u> results.

RECOMMENDATIONS

Based upon these liposome entrapment studies we recommend screening three to six of the most promising liposome preparations. Firstly, preparations made similar to our first preparations which gave 1-3% entrapment should be evaluated. There would be two types in this category-one made with the drug incorporated in the swelling solution and one with the drug incorporated into the original lipid solution. These all would be made to contain 104 mg/ml WR6026·2HCl.

Secondly, preparations made like the aforementioned except with a 10 mg/ml concentration of WR6026·2HCl would be interesting to test. If 10-15% entrapment were achieved the loss of drug unentrapped would be decreased. Of course, the same mg/kg dose of drug should be given in all cases which may necessitate more lipid be administered to get the same dose of entrapped drug as was obtained at 104 mg/ml.

Thirdly, the modified liposome formulation made with DCP and WR6026 at 104 mg/ml should be tested in vivo. Since this preparation appears to give very small liposomes, it may not be as effective as the other liposome systems since the liver or other organs where the parasites reside may not efficiently pick up the smaller liposomes. Certainly, if entrapment is any indication of efficacy, the 15+% entrapped system should be quite effective. If nothing else the higher entrapment may permit lower doses of lipid to be administered to achieve a particular dose of WR6026. We also recommend testing the liposome system which gave 20+%

entrapped with 5 mg/ml since this preparation did not involve DCP and thus the liposomes are neutral like the liposome system with which Alving obtained the best in vivo results.

Beyond these, many more liposome systems could be prepared for evaluation, if desired. We recommend that some biological testing be carried out at this time to narrow the range of formulations for future development.

SOLUBILITY OF WR6026·2 :l IN VARIOUS MEDIA

The solubility of WR6026·2HCl was determined in a variety of aqueous buffer systems and in two alcohols. Excess drug was added to 1-5 ml of medium and sealed in a glass ampule. The ampule was sealed, rotated at least 24 hours in a 37°C bath, the contents centrifuged to remove excess drug and the supernatant liquid analyzed by UV spectrophotometry after appropriate dilution. In a number of solvents the solubility is so high that only the lower limits of the solubility are reported at room temperature.

Table X contains the solubilities or lower limits of solubility obtained. As can be readily seen, WR6026·2HCl exhibits high solubility at pH 6 and below in water and is also quite soluble in ethanol and isopropanol. Based upon the unusual tonicity behavior discussed earlier and these high solubilities, it is quite likely that WR6026·2HCl self-associates in solution.

Table X. Solubility of WR6026·2HCl in Various Media

Solvent	Temperature	Solubility (mg/ml)
H_2O	R.T.[1]	>150
pH 2.0 buffer[2]	R.T.	>50
pH 6.0 buffer[3]	R.T.	>35
pH 9.4 buffer[4]	37°C	0.088
Absoluble Ethanol	R.T.	>50
Isopropanol	37°C	12

[1]R.T. - Room temperature

[2]pH 2.0 buffer - 0.426 Gm of Na_2SO_4 + 0.68 ml of concentrated H_2SO_4 diluted to 500 ml

[3]pH 6.0 buffer - 8.067 Gm of KH_2PO_4 + 1.318 Gm Na_2HPO_4 diluted to 1000 ml

[4]pH 9.4 buffer - 8.295 Gm of Borax ($Na_2B_4O_7$) + 65 ml of 0.1 N NaOH diluted to 1000 ml

STABILITY STUDIES ON WR6026·2HCl IN VARIOUS AQUEOUS MEDIA

When working with WR6026·2HCl, it was noted that aqueous solutions changed color from light yellow to dark amber over a period of time when stored on the laboratory bench. Because of this change, indicating apparent instability, it was decided to carry out a limited stability study.

The composition of the solutions used are given in Table XI. Dilute samples (3.24×10^{-5}M) were placed in ampules and stored under a variety of conditions shown in Table XII. In this phase of the study UV absorption spectra were examined over a period of time for spectral changes.

Table XI. Composition of Solvent Systems Used in the Stability Study of WR6026·2HCl

Solution	Composition
Distilled Water	-
pH 2 Buffer	1.864 gm of KCl and 5.9 ml of 1 N HCl diluted to 500 ml with distilled water
pH 6 Buffer	4.03 gm of KH_2PO_4 and 0.66 gm of Na_2HPO_4 dissolved in enough distilled water to make 500 ml.

Table XII. Storage Conditions for the Qualitative Spectral Change Stability Study for WR6026·2HCl

Temperature	Solvent	Light[a]	Helium Purge
95°	pH 2	No	No
95°	pH 6	No	No
95°	Water	No	Yes
Ambient	pH 2	Yes	No
Ambient	Water	Yes	No

[a]Refers to room fluorescent lighting.

As can be seen in Figures 14-18 and Tables XIII and XIV, significant changes in spectrum occurred in all cases except at pH 2 at room temperature in the presence of light. Solutions also became amber in color as previously noted.

Table XIII. UV Maxima for Samples of WR6026·2HCl Stored in the Dark at 95°C

Time (days)	pH 2	pH 6	Water
0	216, 232, 275s	210, 256, 340	210, 258, 340
20	-	210s, 237, 270	-
30	-	-	210, 254 (purge)
53	255	-	-

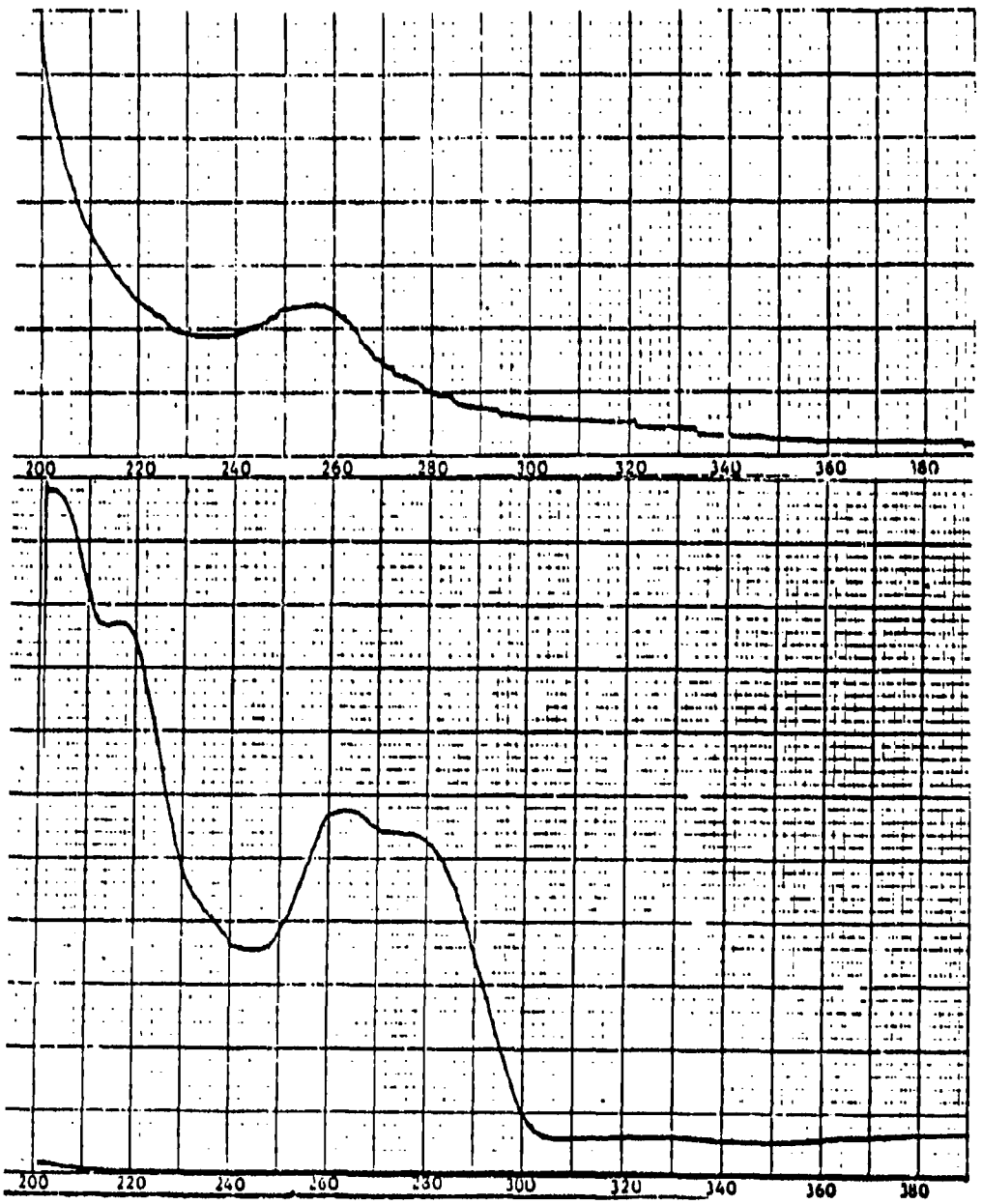

Figure 14: UV Spectrum of WR6026·2HCl. (Conditions: 95°C, no light, pH 2). Lower spectrum is at 0 days. Upper spectrum is at 53 days.

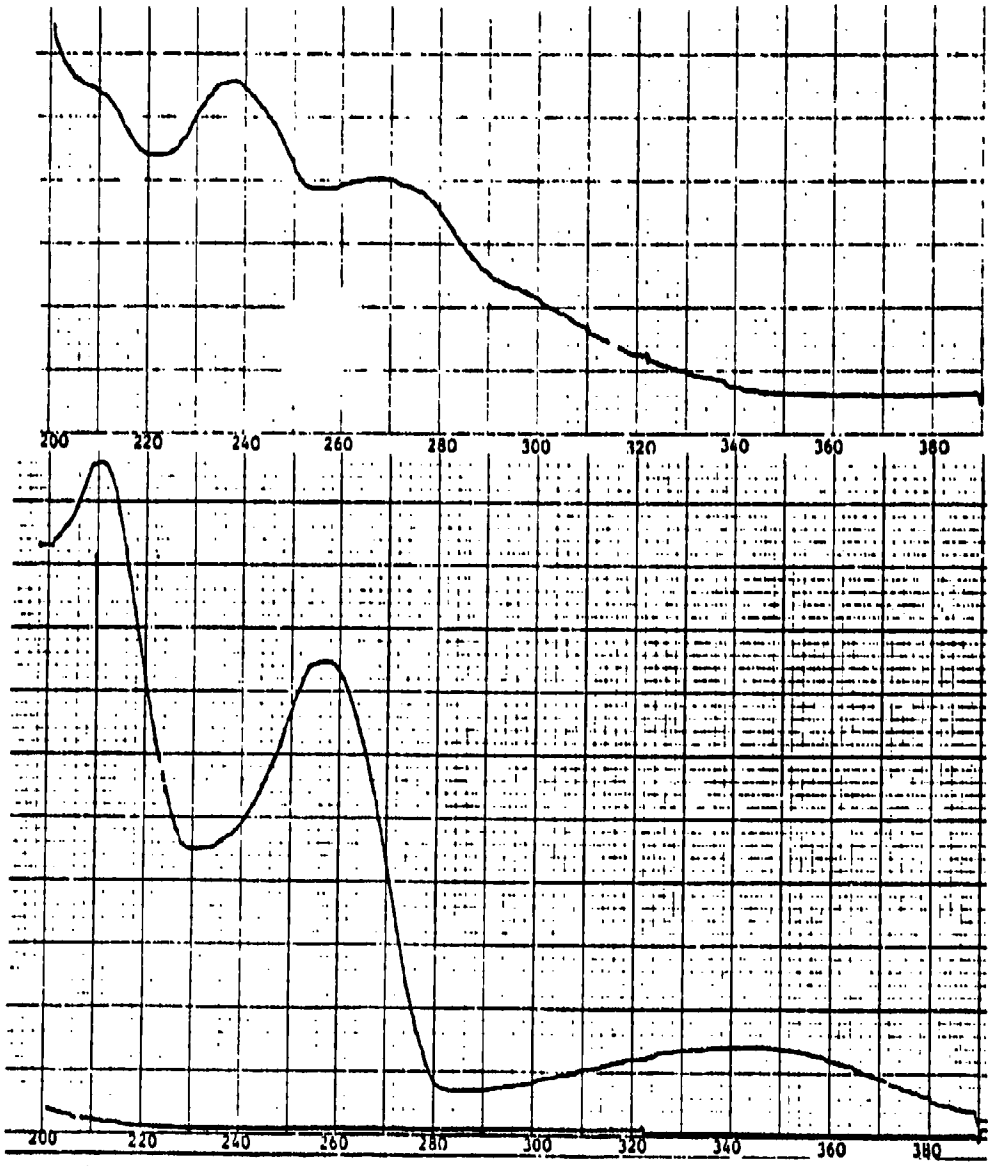

Figure 15: UV Spectrum of WR6026·2HCl. (Conditions: 95°C, no light, pH 6). Lower spectrum is at 0 days. Upper spectrum is at 20 days.

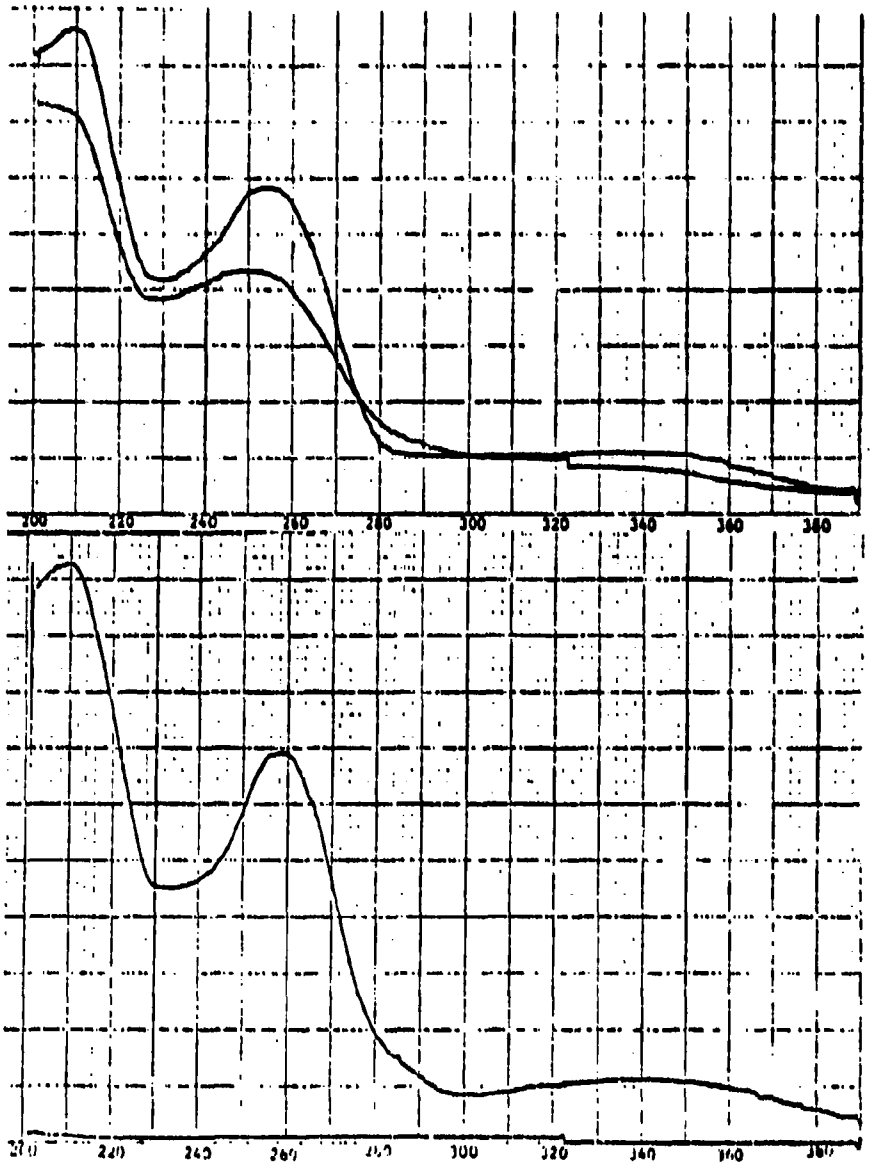

Figure 16: UV Spectrum of WR6026·2HCl. (Conditions: 95°C, no light, distilled water). Lower spectrum is at 0 days. Upper spectrum is at 30 days with the higher curve having a helium purge, while the lower curve did not.

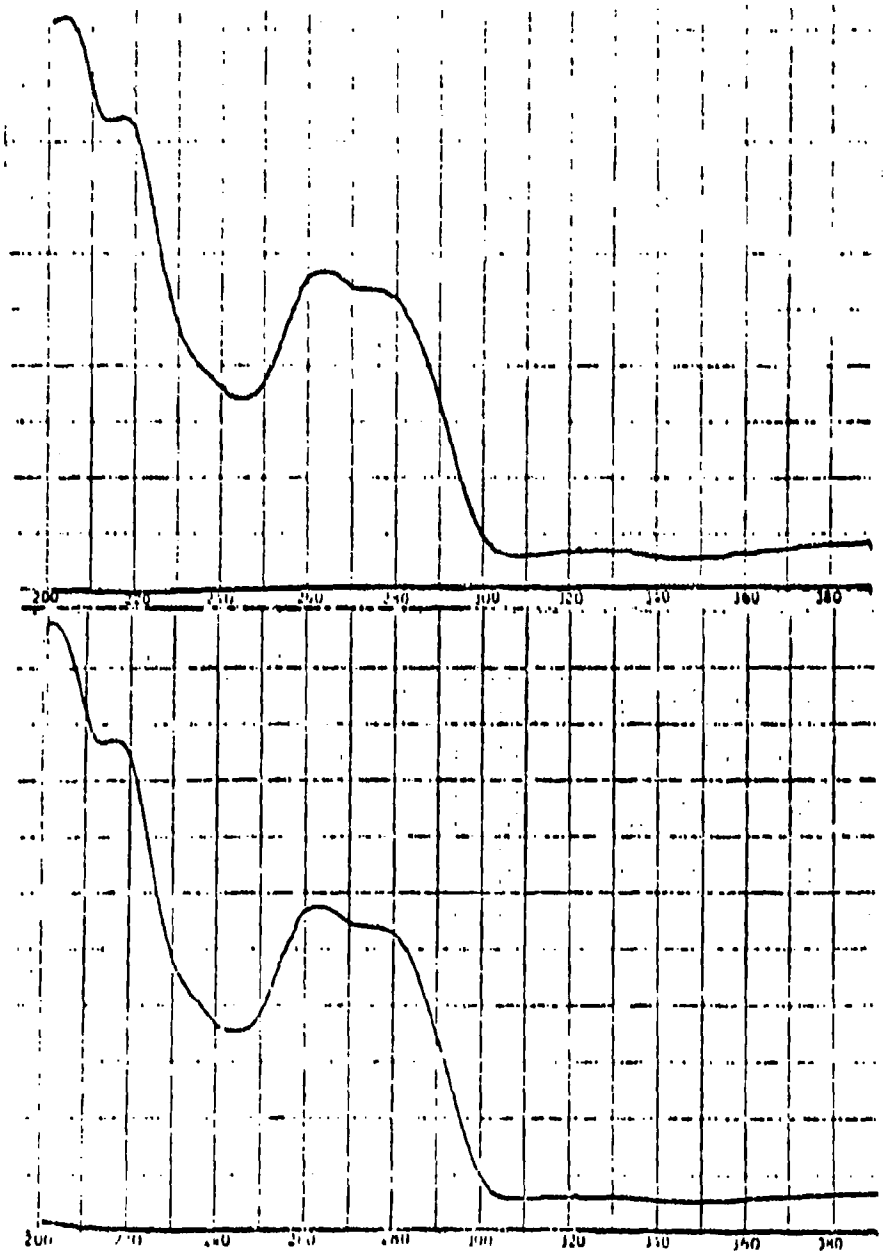

Figure 17: UV Spectrum of WR6026·2HCl. (Conditions: Room temperature, room light, pH 2). Lower spectrum is at 0 days. Upper spectrum is at 20 days.

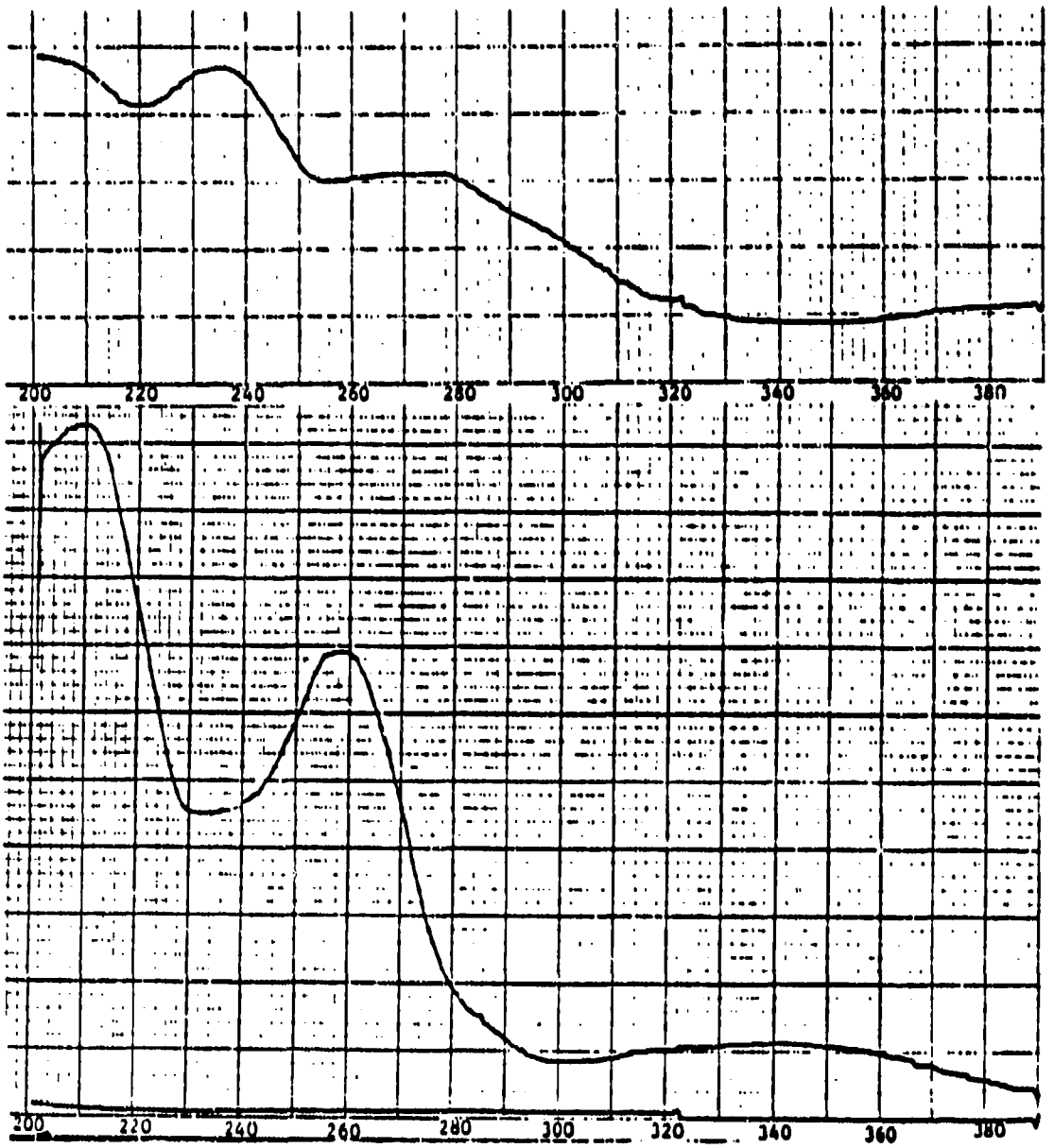

Figure 16: UV Spectrum of WR602b·2HCl. (Conditions: Room temperature, room light, distilled water, no helium purge). Lower spectrum is at 0 days. Upper spectrum is at 20 days.

Table XIV. UV Maxima for Samples of WR6026·2HCl at Room Temperature in the Light

Time (days)	pH 2	Water
0	216, 263, 280s	210, 258, 340
20	216, 263, 280s	236, 278s

Because these spectral changes indicated the possibility of several degradation products, it was difficult to follow the degradation of the parent compound using only UV spectrophotometry. Another study was conducted using high pressure liquid chromatography to follow the disappearance of the parent compound and the appearance of any degradation compounds. The conditions for the HPLC assay are given in Table XV. The standard curve for the assay is shown in Figure 19 and a series of chromatograms as a function of time are shown in Figure 20. Storage conditions for this phase of the study are given in Table XVI.

Table XV. Conditions for the High Pressure Liquid Chromatographic Assay of WR6026·2HCl

Solvent System: 75% methanol/25% 0.01 M phosphate buffer pH 3

Column: Radial Compression Module, 5μ, CN

Flow Rate: 3.0 ml/min

Chart Speed: 0.5 cm/min.

Wavelength: 254 nm

Sensitivity: 0.01 AUFS

Injector: 10 μl Rhedodyne loop

Temperature: Ambient

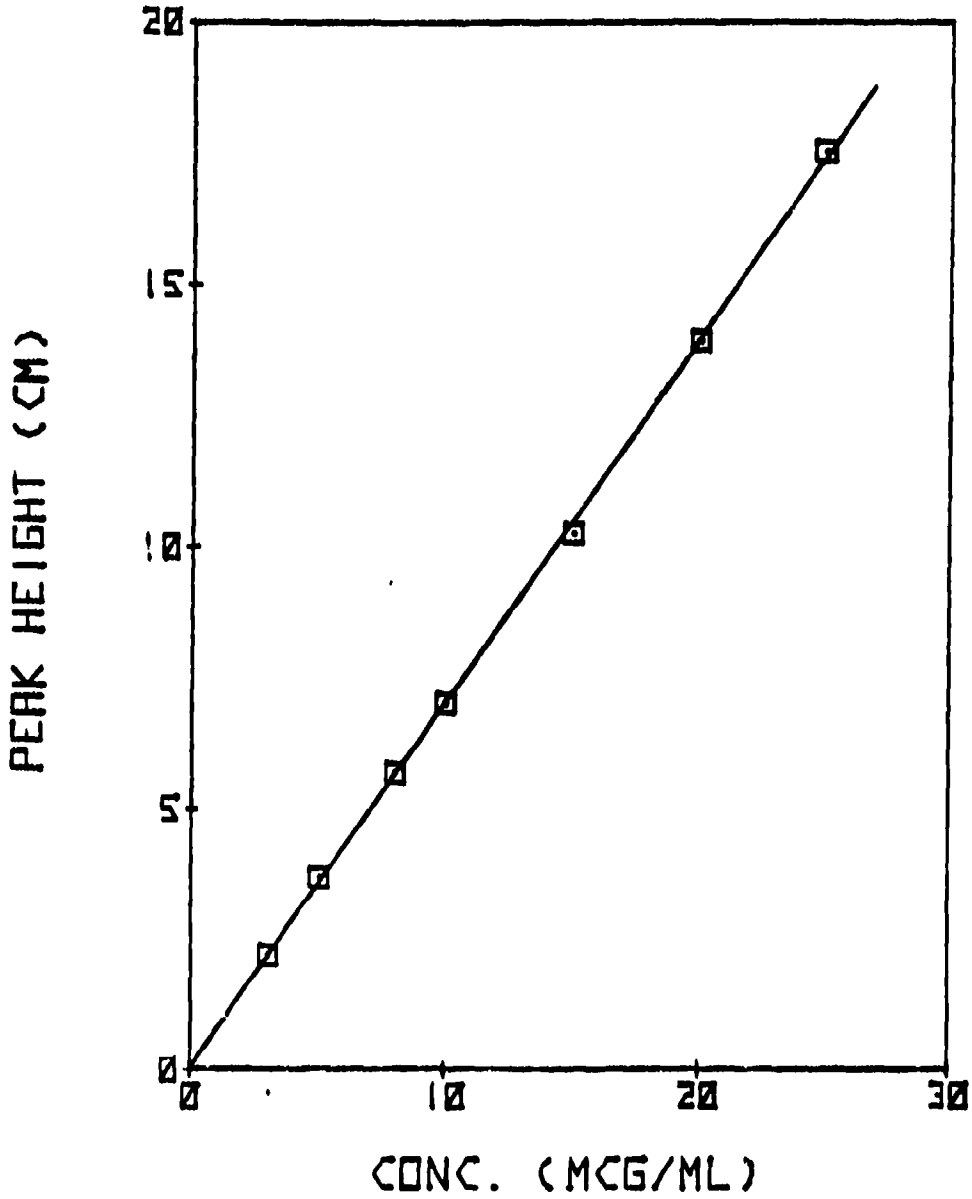

Figure 19: Standard curve for high pressure liquid chromatographic assay for WR6026·2HCl.

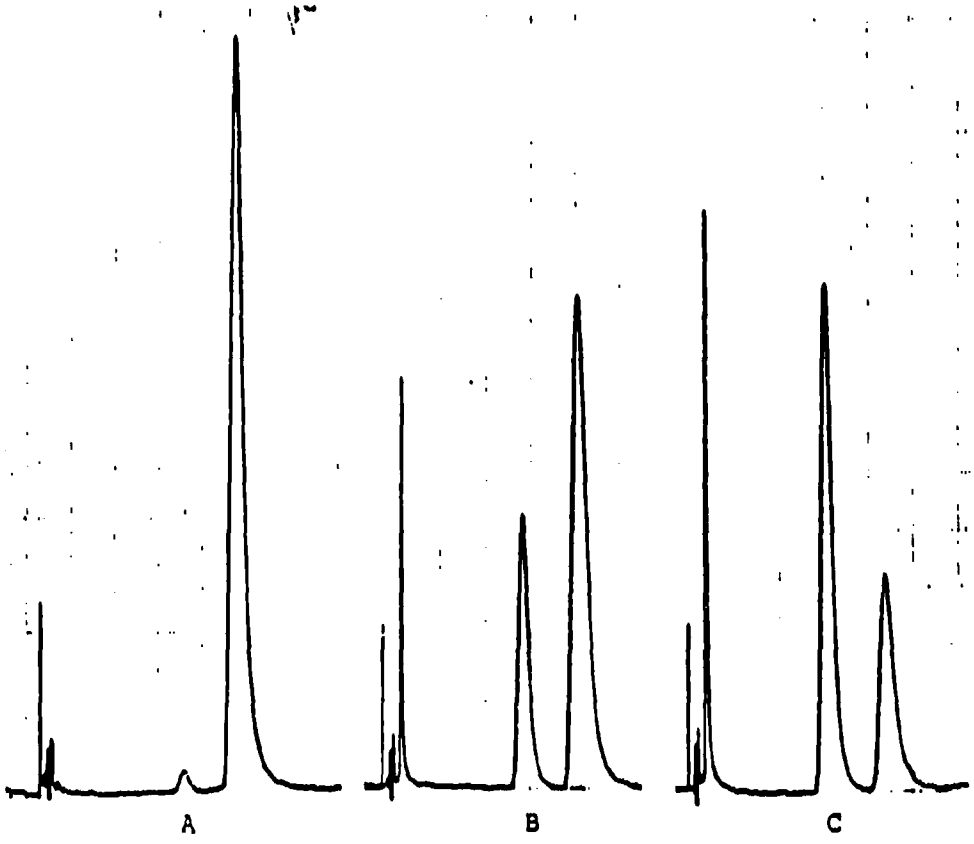

Figure 40: High pressure liquid chromatogram for WR6026·2HCl showing instability. Conditions: pH 2, 95°C, no light, purged with nitrogen. A: 0 hours; B: 24 hours; C: 96.5 hours.

Table XVI. Storage Conditions for the Stability Study of WR6026·2HCl Assayed by High Pressure Liquid Chromatography

Temperature	Solvent	Light[a]	Purge[b]
30°C	pH 2	Yes	Yes
30°C	pH 6	Yes	Yes
30°C	Water	Yes	Yes
95°C	pH 2	No	Yes
95°C	pH 6	No	Yes
95°C	Water	No	Yes

[a]Refers to room fluorescent lighting.

[b]Samples were split with half receiving a nitrogen purge and half receiving no purge.

It is apparent that WR6026·2HCl has a retention time of about 8.8 minutes. Initially, only trace amounts of unknown degradation products are present with retention times of approximately 0.9 and 6.5 minutes. Both degradation product peaks increase with time at the expense of the WR6026·2HCl peak at 8.8 minutes. Thus it appears that this chromatographic method would be suitable to follow the degradation of WR6026.

Plots of the percent of WR6026·2HCl remaining versus time are shown in Figure 21-26. Tables of the percentages remaining are given in Tables XVII and XVIII. The kinetic plots do not appear to follow any simple kinetic order indicating a complex degradation pathway in which the solvent, the presence of oxygen, temperature and light all contribute to the degradation rate. The complexity of the reaction involved can be easily seen by noting that in Table XVII at pH 2, degradation occurs fast relative to the pH 2 solution in Table XVIII. Also is should be noted that the nitrogen

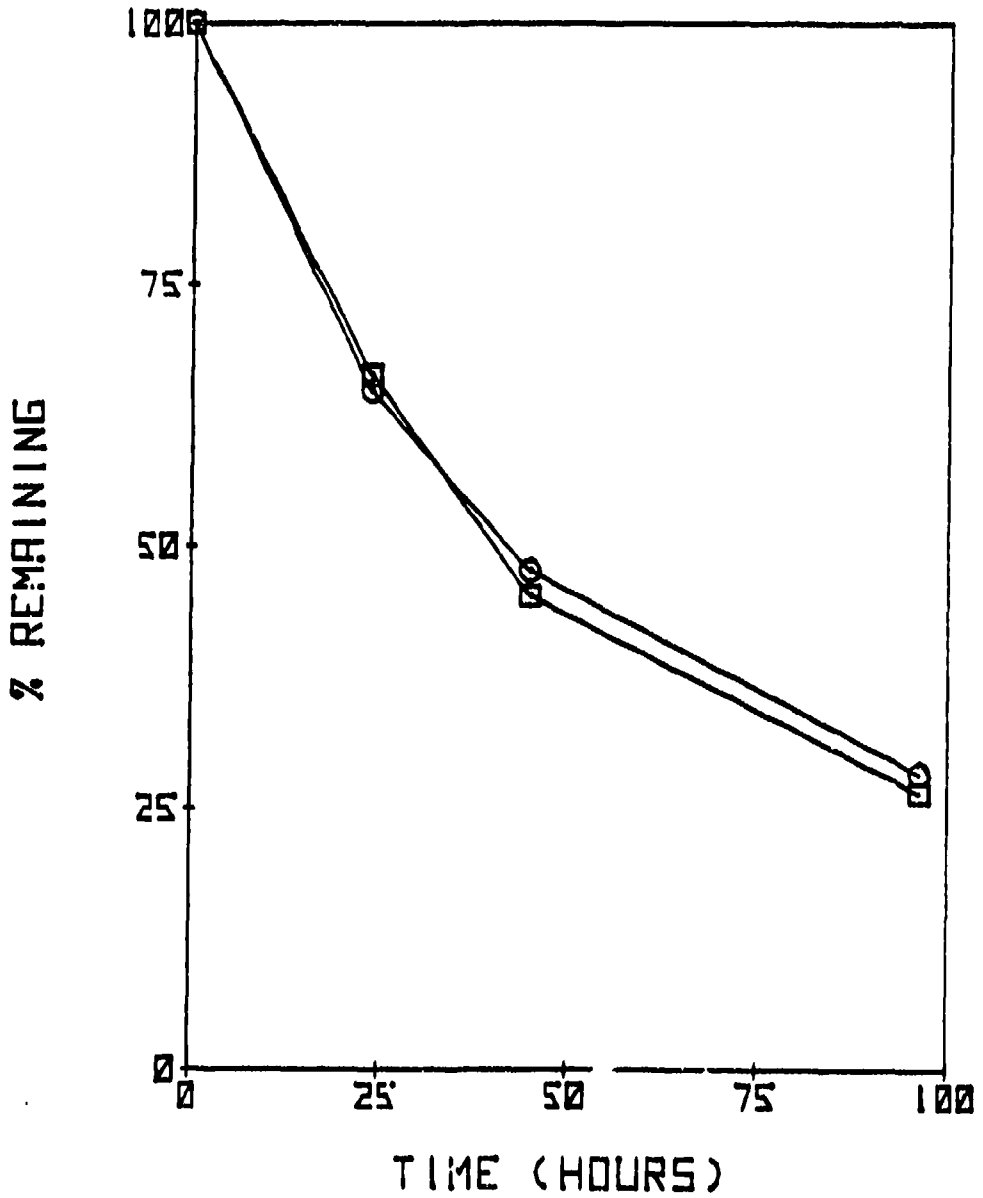

Figure 21: Plot of percent of WR6026·2HCl remaining in pH 2 buffer in the absence of light at 95°C as a function of time. ◘ , no nitrogen purge; ○ , nitrogen purge.

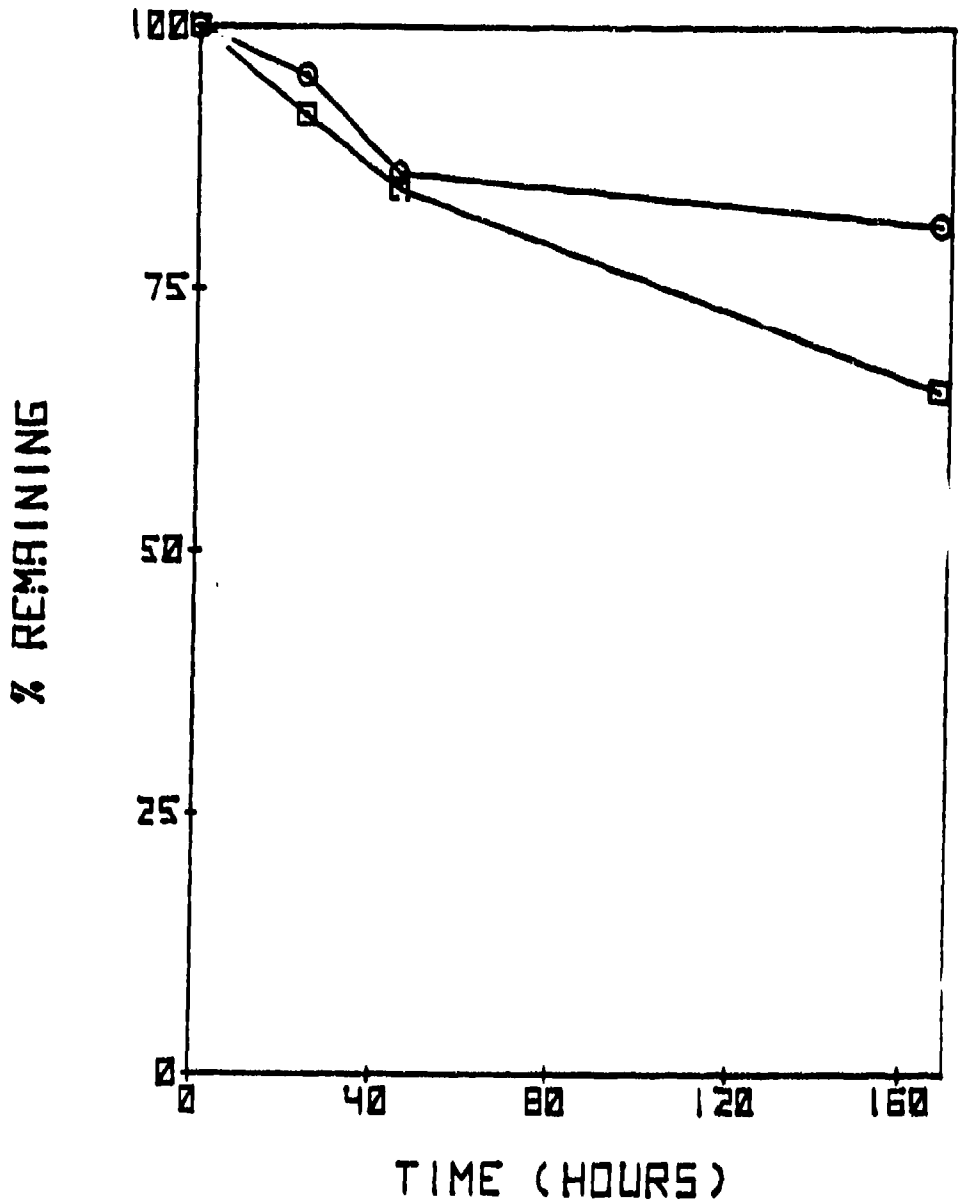

Figure 22: Plot of percent of WR6026·2HCl remaining in water in the absence of light at 95°C as a function of time. ■ , no nitrogen purge; ○ , nitrogen purge.

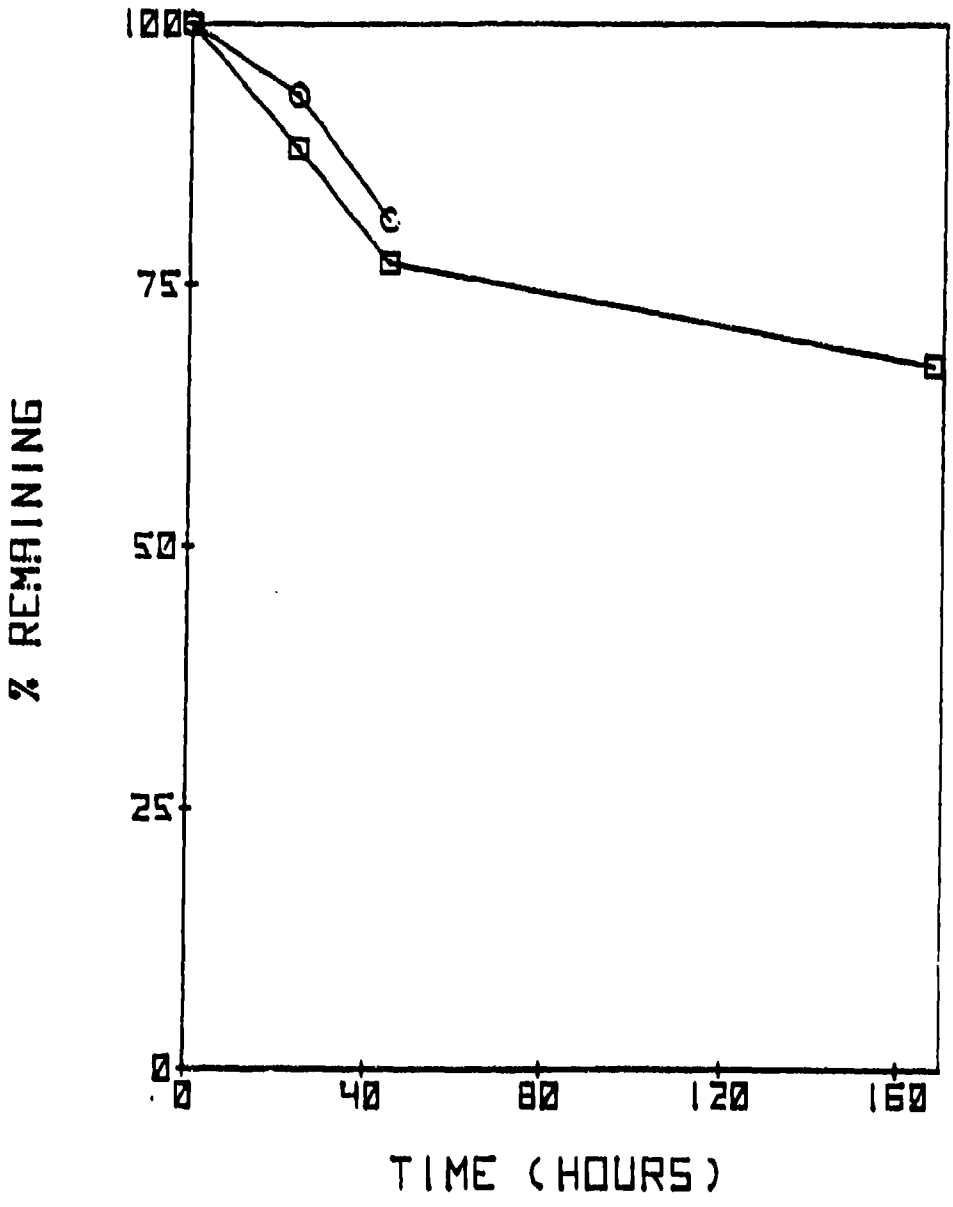

Figure 23: Plot of percent of WR6026·2HCl remaining in pH 6 buffer in the absence of light at 95°C as a function of time. ◘ , no nitrogen purge; ● , nitrogen purge.

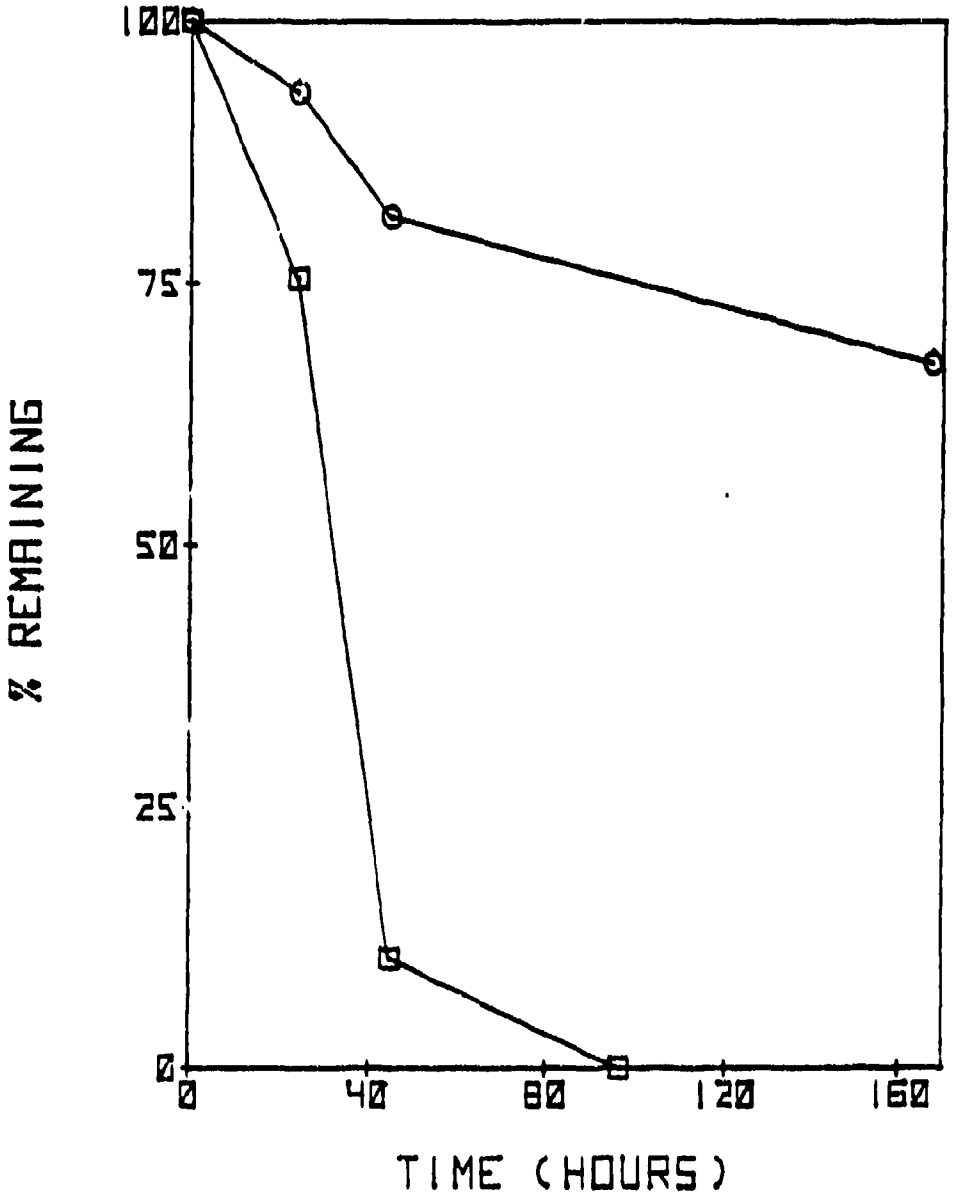

Figure 24: Plot of percent of WR6026·2HCl remaining in pH 6 buffer and light at 30°C as a function of time. ◘, no nitrogen purge; ●, nitrogen purge.

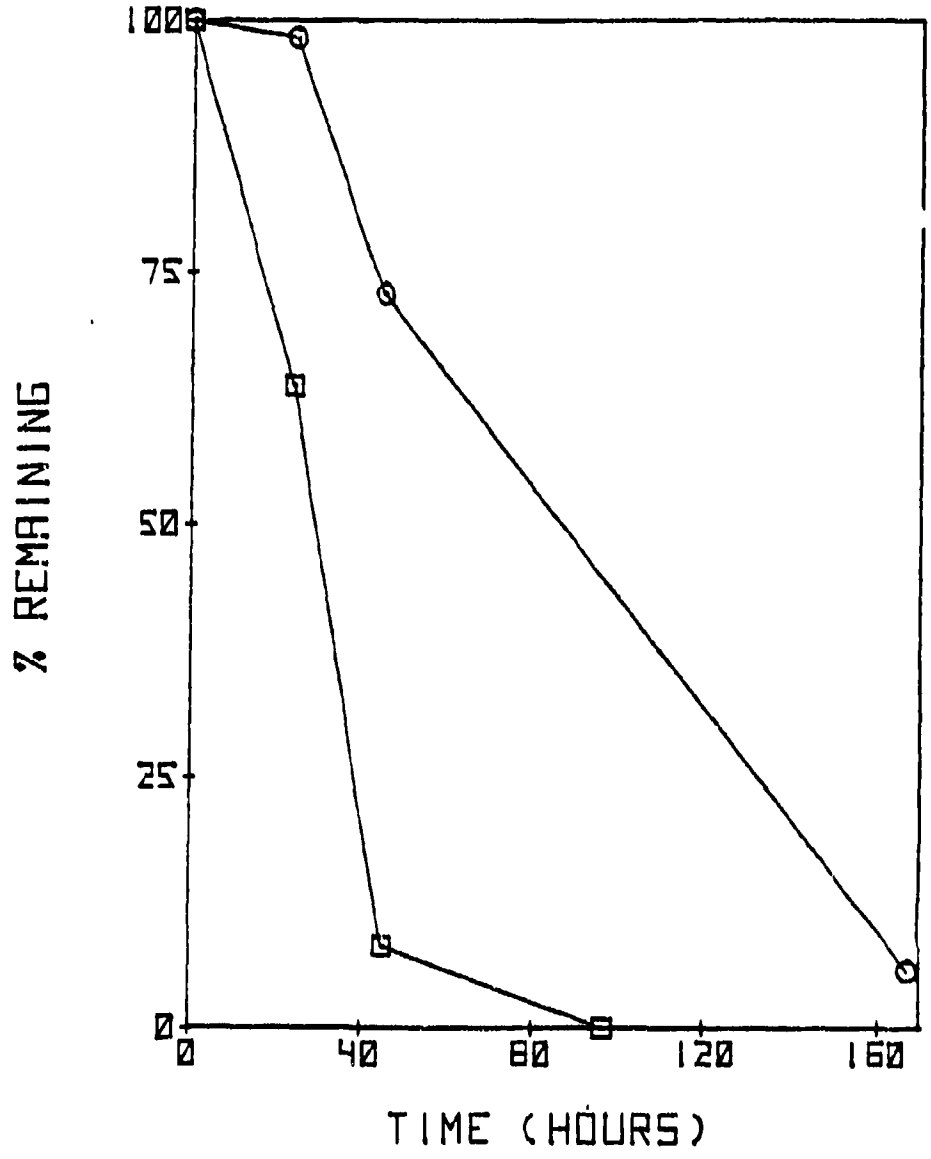

Figure 25: Plot of percent of WR6026·2HCl remaining in water and light at 30°C as a function of time. ◘ , no nitrogen purge; ● , nitrogen purge.

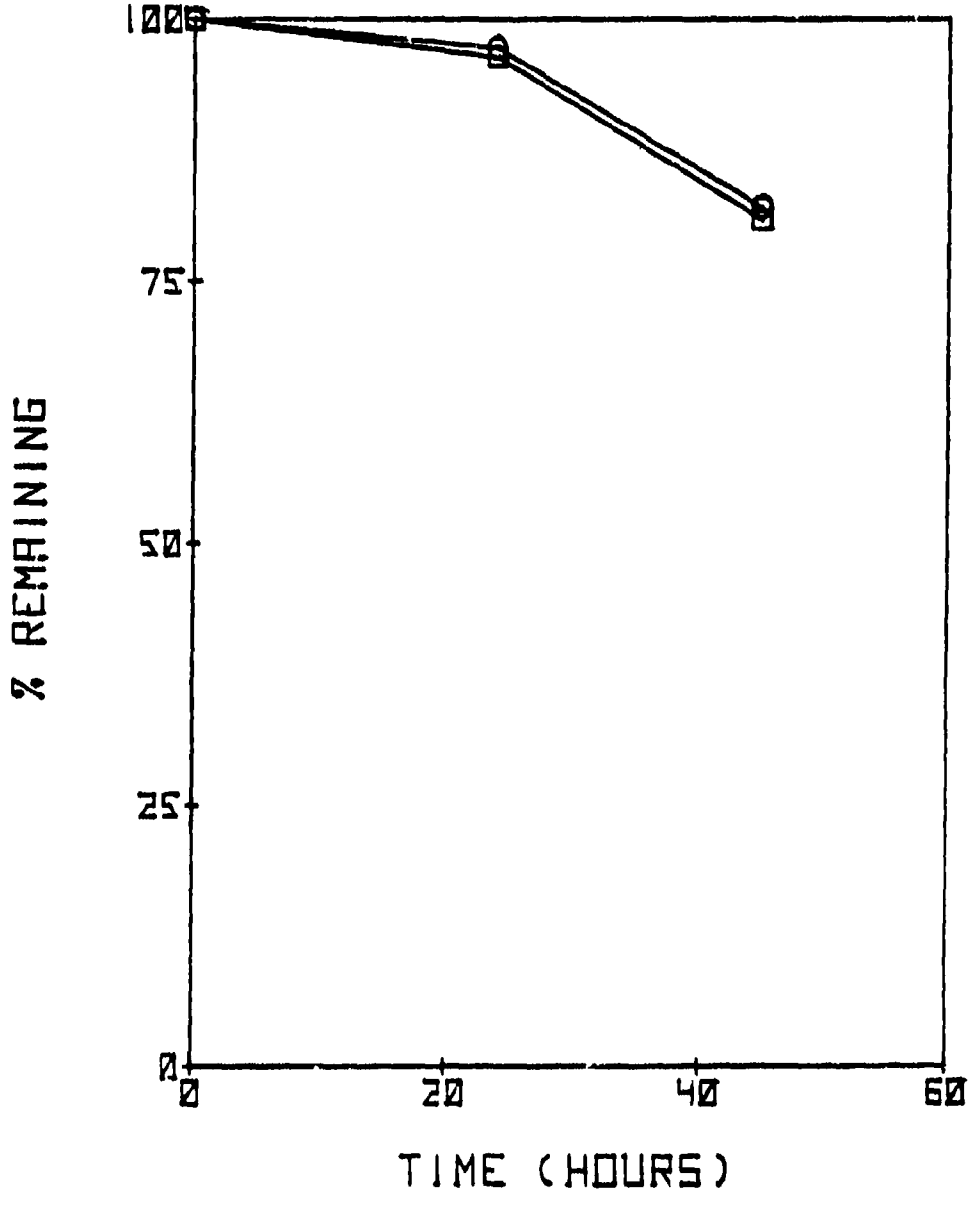

Figure 26: Plot of percent of WR6026·2HCl remaining in pH 2 buffer and light at 30°C as a function of time. ■, no nitrogen purge; ● , nitrogen purge.

purge makes little difference at both pH 6 and in the water solution in Table XVIII, but in Table XVII under these conditions, the nitrogen purge appears to slow the degradation process.

Table XVII. Percent of WR6026·2HCl Remaining as a Function of Time in the Absence of Light at 95°C

	pH 2		pH 6		Water	
Time (hrs)	N_2 purge	No N_2 purge	N_2 purge	No N_2 purge	N_2 purge	No N_2 purge
0	100	100	100	100	100	100
24	64.8	66.4	93.1	88.0	95.4	91.6
45	47.7	45.3	81.2	77.1	86.1	84.6
96.5	28.3	26.5	-	-	-	-
167.5	-	-	-	67.3	81.1	65.3

Table XVIII. Percent of WR6026·2HCl Remaining as a Function of Time in Light at 30°C

	pH 2		pH 6		Water	
Time (hrs)	N_2 purge	No N_2 purge	N_2 purge	No N_2 purge	N_2 purge	No N_2 purge
0	100	100	100	100	100	100
24	97.3	96.4	93.2	75 5	98.3	63.7
45	82.1	80.9	81.4	10.5	72.8	8.1
96.5	-	-	-	0	-	0
167.5	-	-	67.3	-	5.7	-

While no detailed kinetic analysis can be obtained from this limited stability study, it does indicate that a more comprehensive stability study is warranted to establish the optimal storage conditions for WR6026·2HCl solutions. This will be particularily important for any parenteral solutions of WR6026·2HCl and for liposome formulations. At present,

it is apparent that WR6026·2HCl is most stable in the absence of oxygen and light. The pH dependency appears to be reversed between high and low temperature and remains to be investigated in more detail.

COBALT SALT STUDIES

Solubility Studies

Cobalt salt solutions have been found to be potential antidotes for cyanide toxicity (9, 10). In Europe, cobalt edetate (Co_2EDTA) has been accepted as a standard therapy for cyanide intoxication (10). Other cobalt salts have been employed in antidotal therapy with varying degrees of success (9).

In preparation for toxicity and efficacy studies of cobalt salts for the treatment of cyanide poisoning, we have determined the solubilities and saturation pH values for a number of common cobalt salts. In addition, we determined the pH after autoclaving the saturated solutions to determine their stability.

The cobalt concentration was determined using a colorimetric assay (11) which employed nitroso R salt (3-hydroxy-4-nitroso-2,7-naphthalene-disulfonic acid, disodium salt). This assay involves the formation of a red complex between cobalt and nitroso-R salt and has been an accepted method for assaying cobalt for many years (12). In Figure 27, is presented the standard curve for this method employing three different wavelengths for the determination, with 410 nm having the

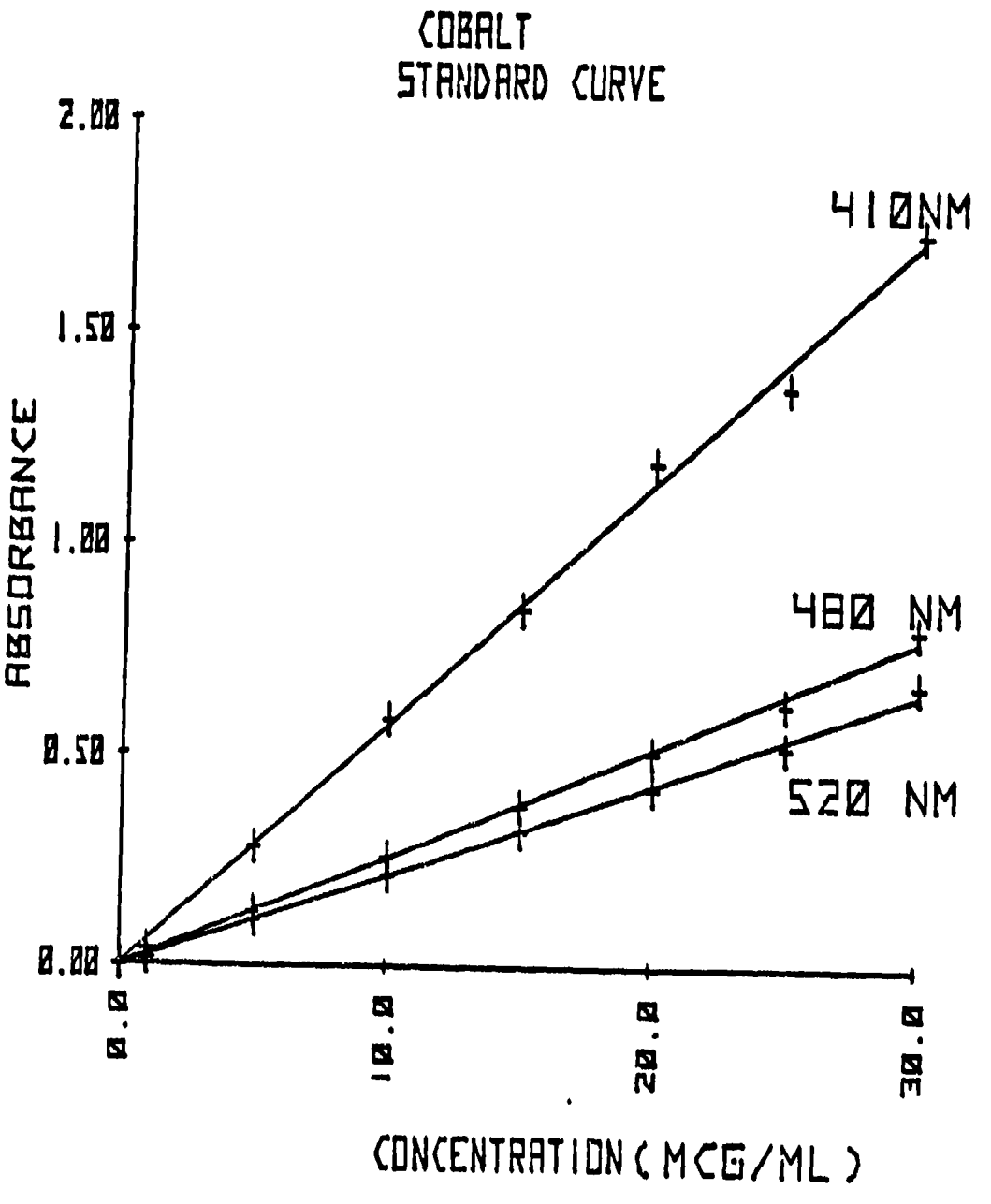

Figure 27: Standard curves at various wavelengths for the colorimetric assay of cobalt(II) ion by the nitroso R salt complex method

highest absorptivity and 480 and 520 nm having about equal absorptivities. In our assay of cobalt salt solubilities, the concentration was calculated using all three wavelengths and the results averaged.

Table XIX shows the solubility and pH data on five cobalt salts. The solubility is given both in of gm/ml Co(II) concentration and cobalt salt concentration. Cobaltous chloride·$6H_2O$ and cobaltous sulfate·$7H_2O$ have exceptionally high solubilities, which accounts for the low pH of their saturated solutions. Only cobaltous chloride shows a significant pH change after autoclaving while the sodium cobaltinitrite solution in an ampoule explodes in the autoclave probably due to the release of NO_2 as a gas.

It was suggested that a nominal parenteral concentration of cobalt salt be 20% for initial toxicity studies. If cobalt chloride is taken as the typical cobalt salt, a 20% solution would be approximately 5% in cobalt ion concentration. Therefore to compare the toxicity and efficacy of various cobalt salts, it is recommended that all salt concentrations be adjusted to give a concentration of cobalt equivalent to 5% (i.e., 50 mg/ml). Thus the actual cobalt salt concentrations will vary but will be equivalent in terms of cobalt concentration. Of the five cobalt salts evaluated, the chloride, acetate, sulfate and cobaltinitrite salts have sufficient solubility to give 5% cobalt concentrations. Parenteral solutions of these cobalt salts have been forwarded to WRAIR for further evaluation. The concentrations of each

TABLE XIX. Cobalt Salt Properties in Aqueous Solution (37°C)

Cobalt(II) Salt	Solubility		pH
	Co(II) Conc (G/ml)	Salt Conc (G/ml)	
Chloride·$6H_2O$	0.2685	1.084	2.56(1.49)*
Acetate·$4H_2O$	0.0970	0.410	6.19(6.29)
Formate·$2H_2O$	0.0129	0.398	6.85(6.68)
Sulfate·$7H_2O$	0.1542	0.7355	3.31(3.29)
$Na_3Co(III)(NO_2)_6$	0.0581	0.3982	7.73(ampule explodes in autoclave)

*pH values in parentheses are taken after autoclaving the saturated solution

salt to achieve 5% cobalt concentrations are shown below:

	mg/ml
Cobalt Chloride·$6H_2O$	201.9
Cobalt Acetate	211.3
Cobalt Sulfate·$7H_2O$	238.5
Sodium Cobaltinitrite	342.7

Even though all salt solutions except the cobaltinitrite, can be autoclaved without significant change, we will use microfiltration under a laminar flow hood to prepare the initial test batches of these parenteral solutions.

It should be noted that these cobalt salt solutions will be extremely hypertonic and will most likely be painful upon intramuscular injection and possibly cause tissue damage. In addition, a high level of cobalt ion may be quite toxic itself. Complexes of cobalt with EDTA, ammonia, etc. or weakly dissociated salts such as gluconate, benzoate or lactate may be less toxic and cause less tissue damage. Studies have been initiated to investigate the solubility of various complex cobalt salts and the weakly dissociated cobalt salts to determine if they offer any advantages over the previously studied cobalt salts.

Cobalt Salt Solutions for Parenteral Toxicological Evaluation

The following cobalt salt solutions were prepared for toxicological evaluation. All concentrations were calculated to be equivalent in cobalt ion concentration which was set at 5% (0.8484 M).

The two solutions containing tetrasodium EDTA were prepared to give equal molar (0.8484 M) and half equal molar (.424 M) concentrations of EDTA with respect to the cobalt ion concentration.

All solutions were prepared in Sterile Water for Injection and microfiltered through a 0.22 micron membrane filter under a laminar-flow hood. Each vial contains 10.1-10.2 ml of the respective cobalt salt solutions.

Cobalt Salt Solution	Molecular Weight	Conc. (mg/ml)
1. Cobalt Chloride Hexahydrate ($CoCl_2 \cdot 6H_2O$)	237.93	201.9
2. Cobalt Acetate Tetrahydrate ($Co(OAc)_2 \cdot 4H_2O$)	249.08	211.3
3. Cobalt Sulfate Heptahydrate ($CoSO_4 \cdot 7H_2O$)	281.10	238.5
4. Cobalt Nitrate Hexahydrate ($Co(NO_3)_2 \cdot 6H_2O$)	291.04	246.9
5. Sodium Cobaltinitrite ($Na_3CO(NO_2)_6$)	403.94	342.7
6. Cobalt Chloride Hexahydrate ($CoCl_2 \cdot 6H_2O$)	237.93	201.9
Tetrasodium EDTA Dihydrate ($Na_4EDTA \cdot 2H_2O$)	416.2	353.1
7. Cobalt Chloride Hexahydrate ($CoCl_2 \cdot 6H_2O$)	237.93	201.9
Tetrasodium EDTA Dihydrate ($Na_4EDTA \cdot 2H_2O$)	416.2	176.6

PVP COPRECIPITATES OF WR171,669·HCl (HALOFANTRINE HCl)

Since WR171,669·HCl exhibits poor aqueous solubility, poor dissolution rates in in vitro tests and erratic oral bioavailability, it was deemed desirable to determine

whether its dissolution characteristics could be improved. Previous studies have shown that coprecipitation of many drugs with water soluble polymers, in particular, with polyvinyl-pyrrolidone (PVP, Povidione, Plasdone C, Kollidon) can enhance the solubility and hence their dissolution rates (13, 14). The proposed mechanism of this enhancement, which is often over a hundred-fold, centers on the inhibition of crystal growing of the stable crystal form of the drug. By preventing such crystal growth, the drug is trapped in the polymer upon rapid evaporation of the solvent in a "high energy," amorphous or "glossy" state which is thermodynamically more active, resulting in higher solubility and dissolution rates.

Preparation of PVP Coprecipitates

All coprecipitates were obtained by flash evaporating a methanol or other volatile organic solvent solution of WR171,669·HCl with the appropriate amount of PVP[1] to give the desired ratios. The resulting "glossy" coprecipitate was dried futher in a vacuum oven at 60-70°c for 24-48 hours to drive off the last portions of solvent. The dried coprecipitate was powdered and either was used as a powder or compressed into non-disintegrating flat-discs for dissolution studies.

[1]PVP was available as Plasdone® (GAF Corp.) or Kollidon® (BASF/Wyandotte) in various molecular weights.

Powder Dissolution Testing

Two liters of pH 3 HCl was placed in a glass beaker which in turn was placed in a 37°C water bath. A three-inch, half-moon-shaped paddle was used for agitation and was fixed approximately 1¼ inches from the bottom of the container and the stirring was controlled at 120 rpm. The pH 3 medium was employed to duplicate the medium used in previous dissolution studies by $INTER_X$ (15).

An initial 5 ml blank sample was replaced with 5 ml of pH 3 medium. The coprecipitate material (equivalent to approximately 50 mg of WR171,669·HCl) was added in powder form to begin the dissolution test and 5 ml samples were removed at appropriate time intervals for UV assay (258.2 nm, ε = 48,400). Each sample was replaced with an equivalent volume of blank medium.

The dissolution profiles of coprecipitates with PVP (MW 10,000, Plasdone C-15) are shown in Figure 28 and the dissolution data tabulated in Table XX. As can be easily seen, the 1:9 and 1:3 coprecipitates dissolve, as powders, quite rapidly while the 1:1 and 1:2 coprecipitates are significantly slower. While the 1:9 ratio represents only 10% drug content, the 1:3 ratio represents 25% drug content which could offer a dosage form size convenient for oral ingestion.

Non-Disintegrating Tablet Dissolution

Since these data indicated that more controlled studies were warranted on the PVP coprecipitate-WR171,669·HCl system, further dissolution studies were conducted with non-disintegrating

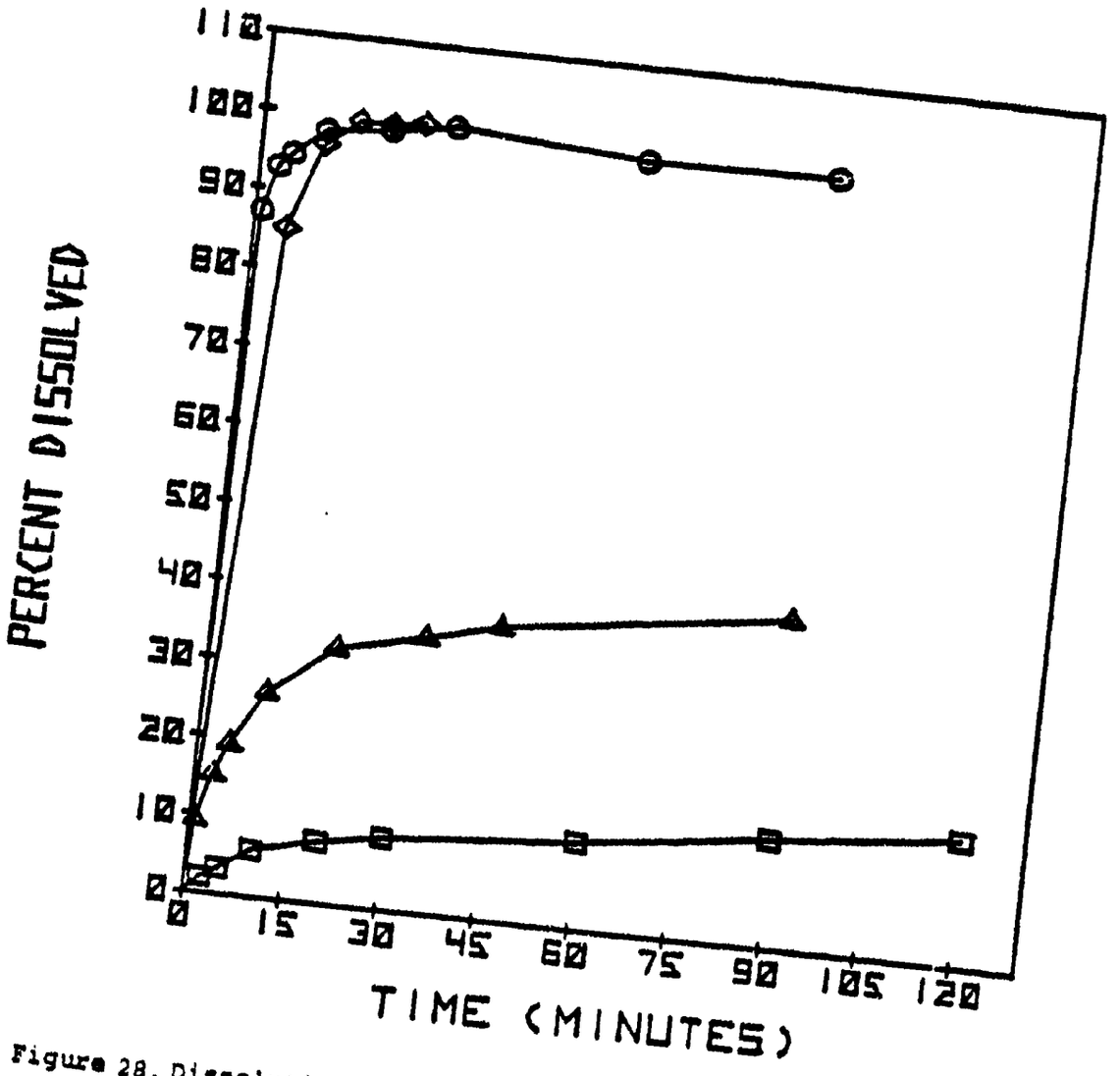

Figure 28. Dissolution Profiles for WR171,669·HCl/PVP Coprecipitates in pH 3 HCl at 37°C using Plasone C-15 (MW-10,000).

Key: ⊡, 1:1 Drug/PVP Coprecipitate
△, 1:2 Drug/PVP Coprecipitate
○, 1:3 Drug/PVP Coprecipitate
◇, 1:9 Drug/PVP Coprecipitate

Table XX. **Percent Dissolved from WR171,669·HCl/PVP** (Plasdone C-15) Coprecipitates in pH 3 HCl at 37°C

Time (min)	1:1	1:2	1:3	1:9
0	0	0	0	0
1	-	9.3	87.1	-
2.5	2.1	-	-	-
3	-	15.2	92.9	-
5	3.3	19.2	94.7	85.2
10	6.0	26.1	98.0	96.3
20	7.9	32.6	98.7	99.5
30	9.2	-	100.0	-
33.5	-	35.1	-	-
45	-	37.4	-	-
60	11.2	-	98.2	-
90	13.9	42.0	98.5	-
120	16.2	-	-	-

tablets. These studies were conducted as dissolution from a constant surface area tablet to avoid the variability that often arises in powder dissolution studies from particle size and wetting effects.

Powdered coprecipitate (300-500 mg) was compressed into a 13 mm diameter flat-faced tablet on a Pasadena Hydraulics Press employing a KBr pellet press.

The resulting tablets were mounted in a dissolution apparatus (14) containing a flat-bladed paddle stirrer. The dissolution rate of each coprecipitate tablet was determined by sampling from the pH 3 dissolution medium (500 ml). All studies were conducted at 37°C and with a stirring speed of 120 rpm. Assays were performed on withdrawn samples by UV absorption spectrophotometry at 258.2 nm (ε = 48,400).

Figure 29 shows the dissolution profiles of five of the coprecipitates studied. The highest dissolution rate was obtained for a 3:1 (PVP:drug) weight ratio using Plasdone C-15 which has a nominal molecular weight of 10,000. The weight ratio effect is quite dramatic because a drop to 2.5:1 and finally to 2:1 produces a drastic drop in dissolution rate of 65-fold. Increasing the weight ratio to 9:1 also produces a drop in dissolution ratio and thus it appears that the peak in dissolution behavior appears around a 3:1 weight ratio. Changing the PVP molecular weight to 2,500 (K12PF) did not enhance the dissolution rate of the coprecipitate at a 3:1 ratio but this may not be the optimum ratio for this molecular weight PVP. Table XXI summarizes

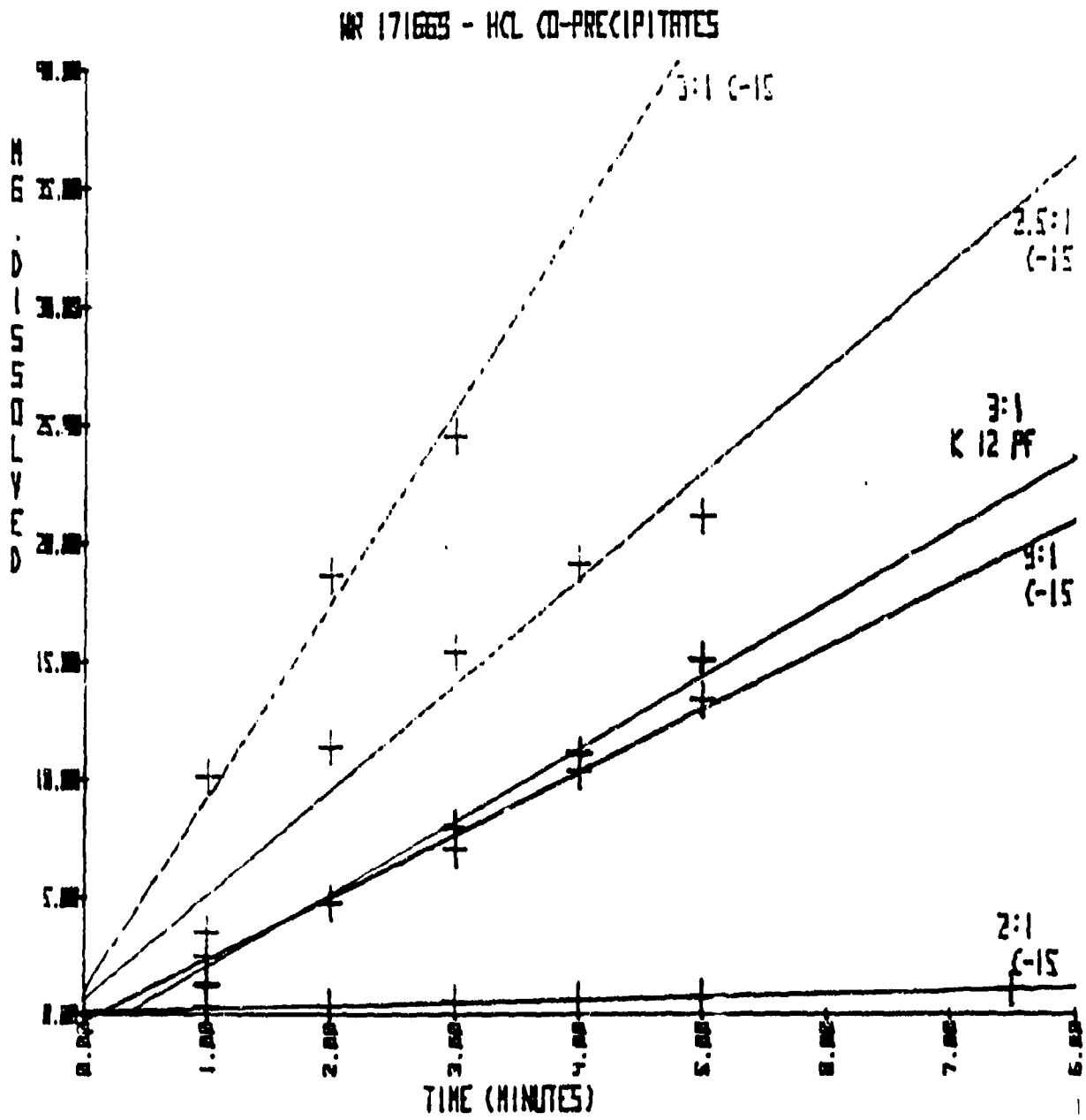

Figure 29. Dissolution Profiles for Various PVP Coprecipitates of WR171,669·HCl from Non-Disintegrating Tablets. Key: C-15 is Plasdone C-15 (MW-10,000); K12PF is Kollidon 12PF (MW-2,500).

the dissolution rate data for these coprecipitates. The pure drug tablet and 1:1 were not shown in Figure 29 because they would fall below the 2:1 line and be essentially parallel to the abscissa over the period represented in the figure.

Table XXI. Dissolution Rates of WR171,669·HCl/PVP Coprecipitates at 37°C in pH 3 Aqueous Medium

PVP Molecular Weight	Weight Ratio (PVP:Drug)	Dissolution Rate (mg/min)
10,000	0:1 (Pure Drug)	0.07
10.000	3.0:1	8.18
10,000	2.5:1	4.44
10,000	1.0:1	0.13
10,000	1.0:1	0.08
10,000	9.0:1	2.63
2,500	3.0:1	3.06

Since the dissolution rate enhancement of these coprecipitates with PVP and WR171,669 are so dramatic we continued our study of their characteristics to determine whether larger dissolution rate enhancements could be obtained.

Further studies on the dissolution behavior of PVP coprecipitates of WR171,669·HCl are shown in Figure 30-33 in which the molecular weight effect of the PVP is investigated more thoroughly. These studies are again with non-disintegrating compressed discs of each coprecipitate.

With the low molecular weight (MW=2,500) PVP (Figure 30) the highest dissolution rate was obtained at a 4:1 ratio while as before the 10,000 molecular weight PVP (Figure 31) exhibits its highest rate at a 3:1 ratio. It should also be

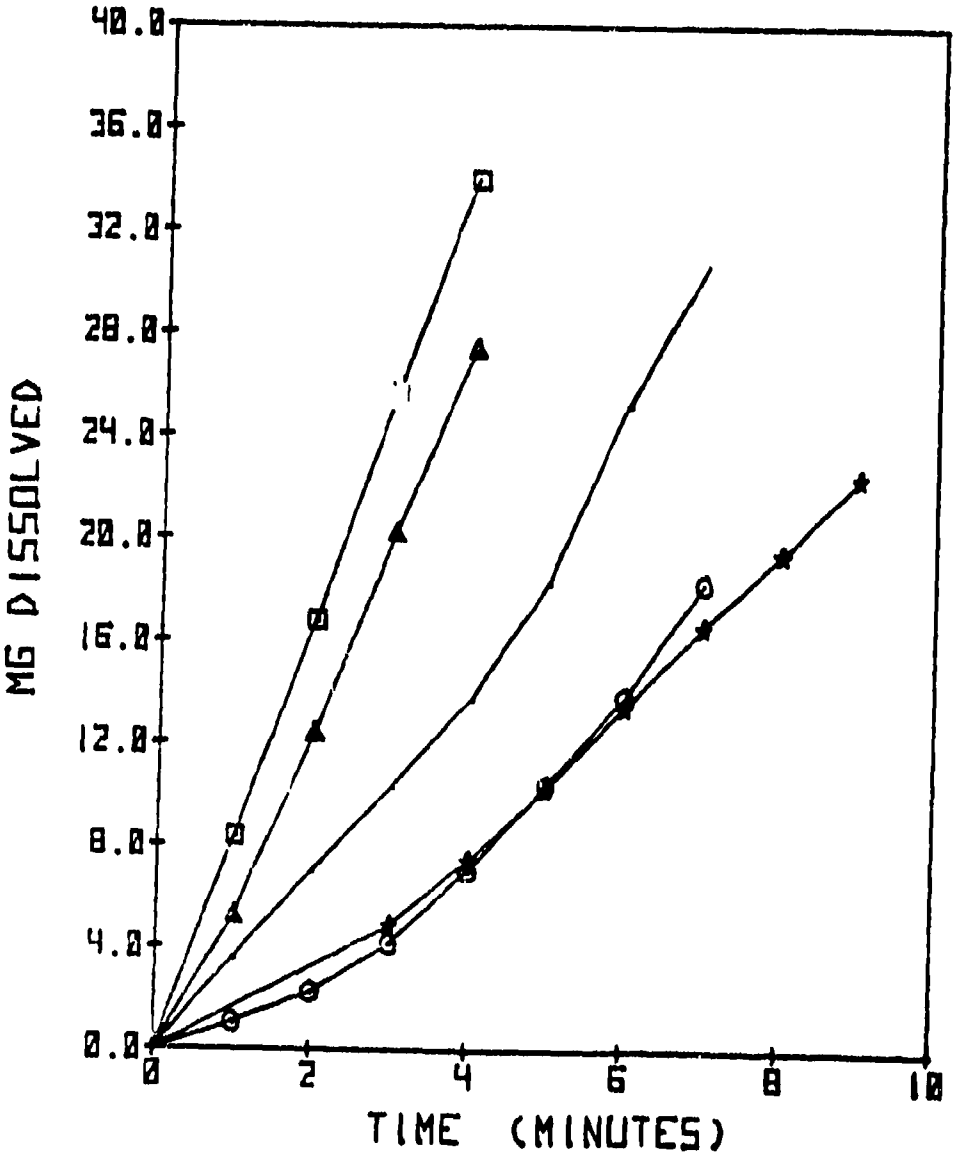

Figure 30: Dissolution Profiles of WR171,669·HCl from Coprecipitates with PVP (MW=2,500, Kollidon® 12PF) at Various Weight Ratios (PVP:Drug) Key: ● , 9:1; □ , 4:1; △ , 3:1; ○ , 2.33:1; ★ , 1.78:1.

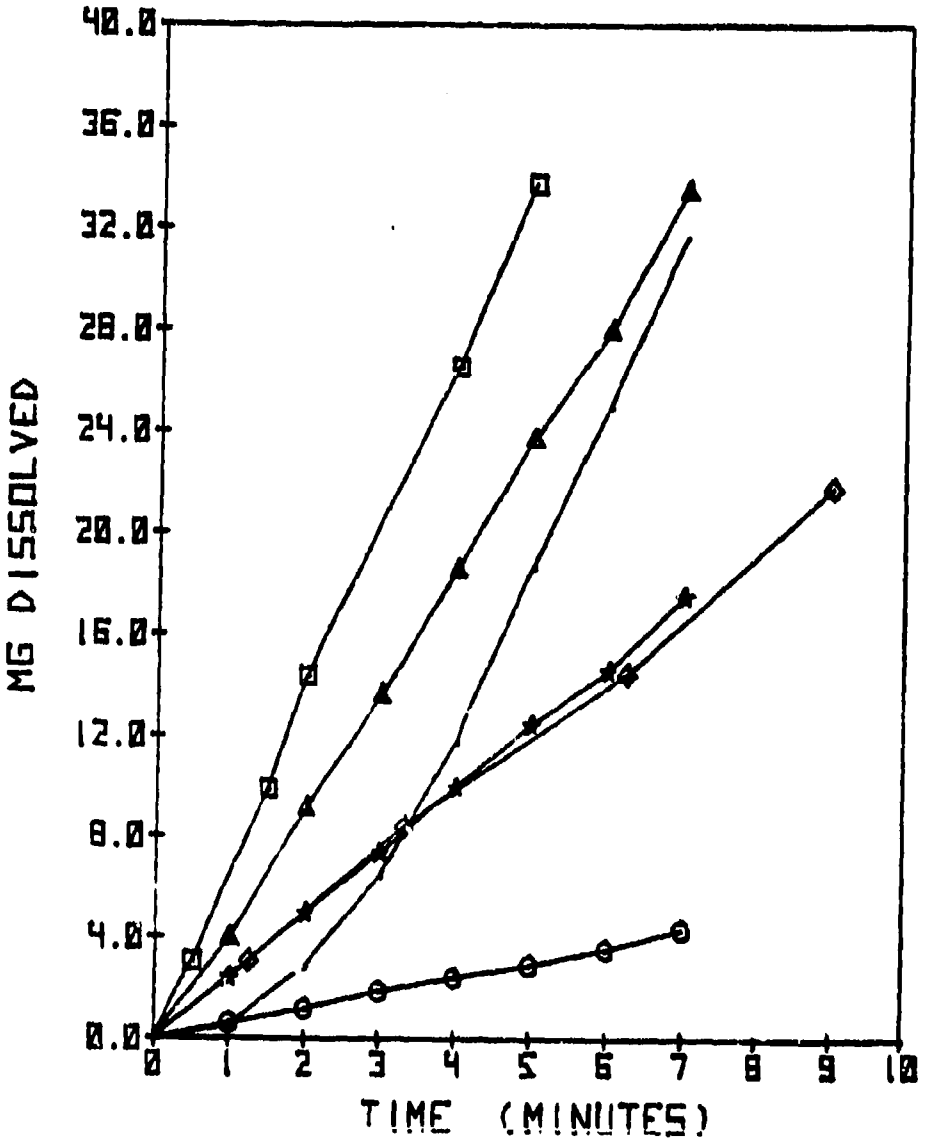

Figure 31: Dissolution Profiles of WR171,669·HCl from Coprecipitates with PVP (MW-10,000, Plasdone® C-15) at Various Weight Ratios (PVP:Drug) Key: ★ , 9:1; △, 4:1; □ , 3:1; ● , 2.33:1, ○ , 1:1; ◇ , 1.5:1:1.5 (CMC, 15 cps).

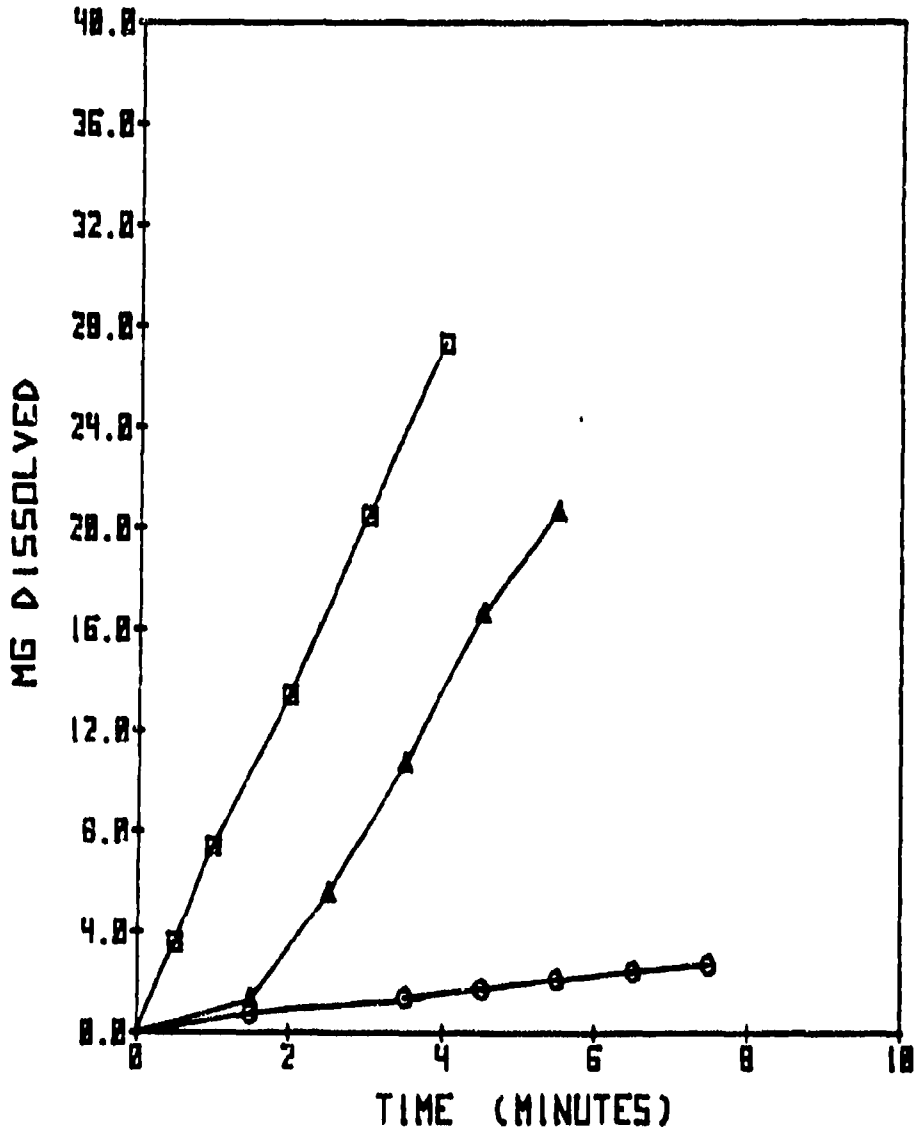

Figure 32: Dissolution Profiles of WR171,699·HCl from Coprecipitates with PVP (MW-11,500, Kollidon® 17PF) at Various Weight Ratios (PVP:Drug) Key: □, 3:1; △, 2.33:1; ○, 1.5:1.

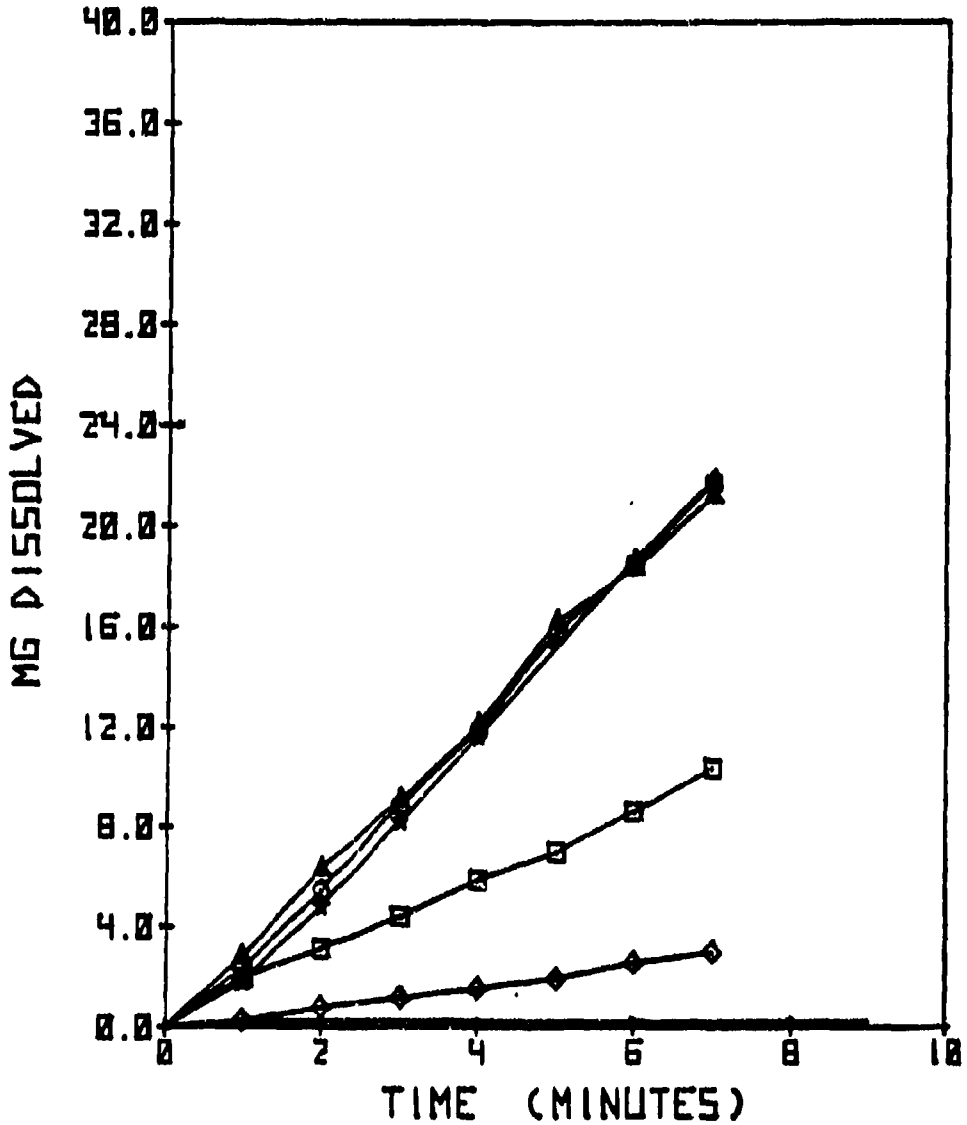

Figure 33: Dissolution Profiles of WR171,669·HCl from Coprecipitates with PVP (MW-40,000, Plasdone® C-30) at Various Weight Ratios (PVP:Drug) Key: □, 9:1; △, 4:1; ○, 3:1; ★, 2.33:1; ◇, 1.5:1; ●, Pure Drug.

mentioned that the dissolution rates of these coprecipitates is quite fast which causes uneven dissolution of the discs which gives rise to the curving dissolution profiles in many cases. A flat constant surface area tablet under sink conditions should give a linear dissolution profile, but if deviations from planarity occur significant upward curvature can be observed.

Slightly higher molecular weight PVP (i.e. MW-11,500 vs. MW 10,000) does not show any significant differences in overall dissolution rate (Figure 32), while 40,000 molecular weight PVP (Figure 33) gives a maximal dissolution rate that is only one-half that obtained with the lower molecular weights. Even a PVP with a molecular weight of 700,000 gives a coprecipitate with an appreciable incrase (>10-fold) in dissolution rate over pure drug.

The molecular weight effect can be seen more clearly in Figures 34 and 35 where the various PVP molecular weights are compared at the same weight ratios. In Figure 35, the lowest molecular weights (2,500, 10,000 and 11,500) give the highest dissolution rates but the higher molecular weights PVPs (25,000 and 40,000) are within a factor of two of these. In Figure 36, 1.5:1 ratios are compared and, even though, their overall dissolution rates are lower than the 3:1 ratios (Figure 35), the higher molecular weight PVP's give the higher dissolution rates. It appears that the higher molecular weight polymers give better protection from nucleation of the stable crystal form of the drug which

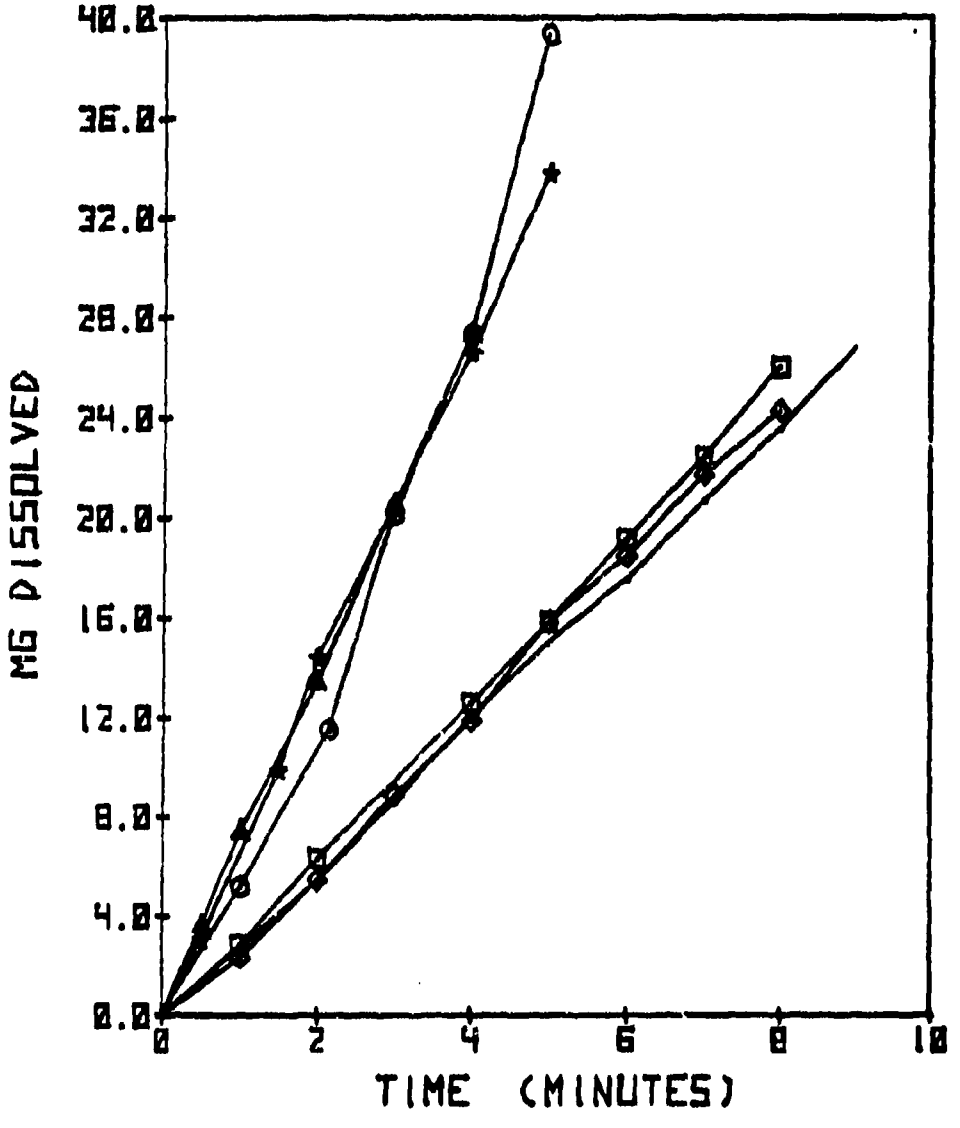

Figure 34: Effect of PVP Molecular Weight on the Dissolution Profiles of WR171,669·HCl Coprecipitates (All 3:1 Ratios) Prepared from Methanol Solutions. Key: ○, MW-2,500; ★, MW-10,000; △, MW-11,500; □, MW-25,000; ◇, MW-40,000 (Plasdone); ●, MW-40,000 (Kollidon).

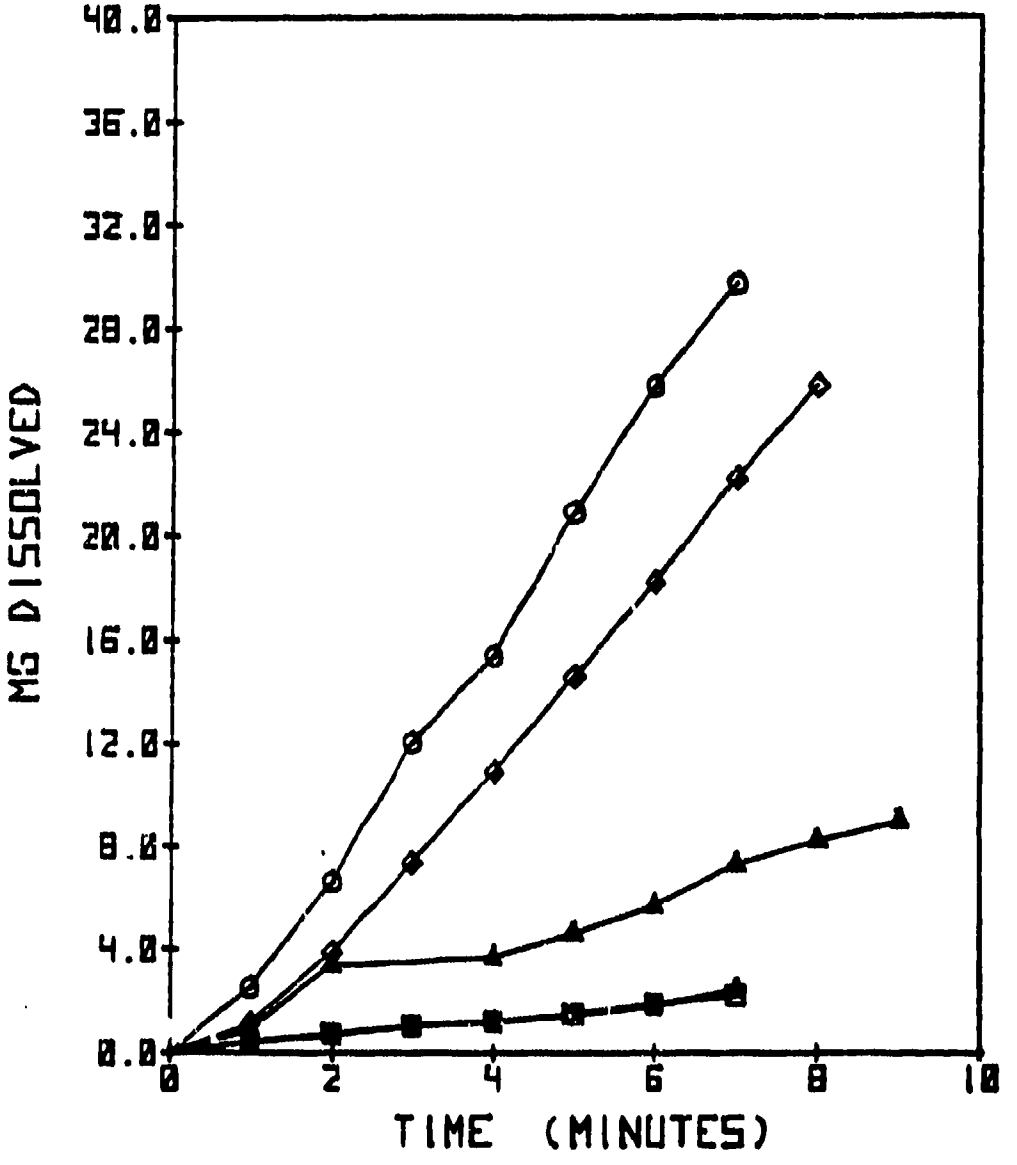

Figure 35: Effect of PVP Molecular Weight on the Dissolution Profiles of WR171 [illegible]·HCl Coprecipitates (All 1.5:1 Rat [illegible]) Prepared from Chloroform Solutions. Key: □, MW-10,000; △, MW-700,000; ○, MW-40,000; ★, MW-2,500; ◇, MW-25,000.

gives rise to the decr[illegible]ase in dissolution. Thus, they are the best crystal [illegible]owth inhibitors but do not give the highest [illegible]olution rates at 3:1 ratios because they have lower diff[illegible]sion coefficients due to their higher molecular weights.

In Figure 36, different coprecipitating solvents are compared (chloroform and methanol). At the optimal ratio of 3:1 the solvent does not have a significant effect. The observed differences can be readily accounted for on the basis of rapid uneven dissolution of the compressed discs.

Thus, it appears clear that a wide variety of PVP molecular weights can be used to prepare satisfactory coprecipitates with WR171,669·HCl to produce dissolution rate enhancements of up to 100-fold.

It should be emphasized that these in vitro results were obtained in large medium volumes which would not be present in vivo. In vivo the supersaturations in GI fluids caused by rapid dissolution may be many-fold and reprecipitation of drug may occur causing little enhancement in absorption.

With these cautions in mind it would be advisable to consider testing one or more of these coprecipitates in an animal model to determine whether these in vitro results are indicative of better absorption rates in vivo. At a 3:1 ratio the drug loading in the coprecipitate is adequate, since for every milligram of WR171,669 to be administered there would be only an addi[illegible]nal 3 mg of PVP (250 mg of drug would require a 1 gram tablet or capsule). It is even

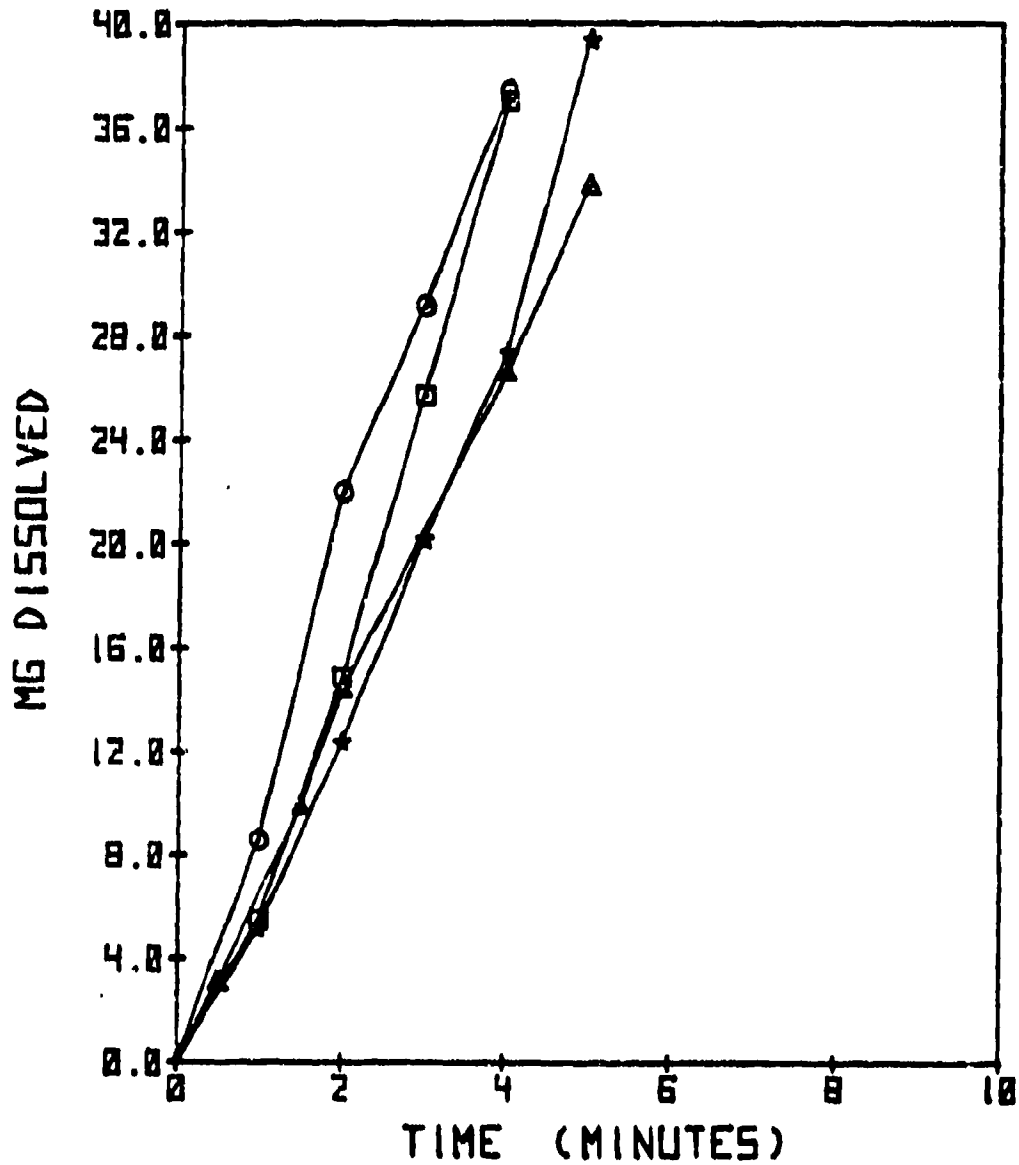

Figure 36: Effect of PVP Molecular Weight and Solvent on the Dissolution Profiles of WR171,669·HCl Coprecipitates (All 3:1 Ratios). Key: □, MW-10,000 (Chloroform); △, MW-10,000 (Methanol); ○, MW-2,500 (Chloroform); ★, MW-2,500 (Methanol).

possible with better absorption of WR171,669 from a coprecipitate that the dose could be reduced which would make the administered formulation size smaller and more easily ingested.

PRODUCTION OF WR171,669·HCl CAPSULES

Capsules of WR171,669·HCl (Halofantrine Hydrochloride) were produced according to the formulation developed by Lafayette Pharmacal Inc. (16). The basic formulation incorporates 250 mg of WR171,669·HCl, 90 mg of Sta-Rx Starch and 7.5 mg of Pluronic L-101 per capsule.

Formulation

The Lafayette formulation is an uncomplicated capsule formulation which includes a diluent (starch) and a wetting agent (Pluronic L101). We employed a commercially available pharmaceutical grade of corn starch (Sta-Rx) which meets all U.S.P. specifications for starch. In addition to being a diluent, this starch will have a disintegrating action to promote deaggregation of the capsule contents upon dissolution of the capsule wall.

The wetting agent, Pluronic L101 (no USP specification), promotes wetting and deaggreation of the capsule contents. According to Lafayette the incorporation of the Pluronic L101 significaintly reduced the disintegration time when compared to capsule formulations without a wetting agent.

Production

Sufficient quantities of WR171,669·HCl, starch and Pluronic L101 were weighed to produce, theoretically, 1500 capsules (See Manufacturing Formulation Appendix).

To coat the WR171,669·HCl with the wetting agent, the Pluronic L101 was dissolved in absolute ethyl alcohol (115 ml)* and incorporated uniformly into the powdered drug. The alcohol was removed by drying at 55°C for four hours in a Colton Model 2433E convection drying oven. The dried mixture was then passed through a 20 mesh screen. Residual moisure (both water and alcohol) was measured by determining the weight change of the powdered formulation after further drying in an Ohaus Moisture Determination Balance. The weight loss after 30 minutes amounted to 0.7%.

The drug-wetting agent mixture was mixed with the starch. In-process assays were performed on three samples of the granulation at this point and then filled into Parke-Davis #0 white opaque capsules (Stock #046-021-999-999-), Lot #TD 0493-2) with a DelTay Capsule Filling Device. The yield of capsules was 1488 (99.2% of theoretical).

Capsules were removed for the following tests: weight variation, disintegration, content uniformity and dissolution.

The finished capsules were packaged in 11 dram amber glass vials with standard closures (Owens-Illinois, PW4011) using a Drug-O-Matic automatic filling machine. The packaged capsule vials were labelled with individual labels numbered 1 through 72. The packaged capsules were shipped by Federal Express to Walter Reed Army Institute of Research.

---

*The ethyl alcohol conforms to all the required tests for USP Dehydrated Alcohol and contains no denaturants or other additivies (see certification, Page 11).

Quality Control Results

The in-process assay was performed by UV analysis (258 nm.) after dissolution in anhydrous methanol and filtration to remove the insoluble excipient (i.e., starch). The in-process assay of the granulation indicated that it was 102.1% of the theoretical amount.

Final assay of ten capsules was performed by dissolving the contents of each capsule in anhydrous methanol and filtering to remove the insoluble excipient (i.e., starch). The methanol solution was assayed by UV analysis (258 nm). The average content was 100.4% of labelled amount with less than a ±10% variation around this value for each capsule, which is well within the USP specification of ±15%.

The weight variation test on 20 capsules gave an average weight of 344.5 mg (theoretical-347.5 mg) which is 99.1% of the theoretical weight. A typical capsule deviated, at most, ±7.5% from this average value which is well within USP limits for capsules.

The disintegration test performed according to USP specifications in 900 ml of distilled water at 37°C gave an average disintegration time of 4 minutes. These results are approximately the same as those obtained by Lafayette when the disintegration apparatus was used with discs.

With a drug as poorly soluble as WR171,669·HCl, a disintegration test is not as meaningful a predictor of bioavailability as a dissolution test. The dissolution test was that employed previously by Interx (17) with a larger

volume of medium i.e., 10 liters as opposed to 3 liters. The medium used was 0.001 M HCl which has a pH of 3. The stirring speed was 120 rpm with a USP paddle stirrer.

The dissolution of two capsules was comparable (see Page 6 of Appendix) but incomplete. There is no indication that this incomplete dissolution is formulation dependent but rather is a reflection of the poor solubility of WR171,669·HCl.

There has been some question as to whether this test is an adequate reflection of bioavailability. In brief, it probably is not, but it does serve as a reasonable comparative test for formulation development. If a lower pH medium (using HCl/KCl) around pH 2 or pH 1 were used, the solubility of WR171,669·HCl would be even lower because of the common ion effect of the increase chloride concentration. With a further reduction in solubility, a lower percentage dissolved would be obtained at long times which would be even more difficult to interpret than our present test conditions. Anytime incomplete dissolution is observed for a solid dosage form, the formulation is often thought to blame but in the case of a poorly soluble drug the formulation may be satisfactory but the intrinsic solubility of the drug will govern how much finally dissolves. We have striven to obtain dissolution conditions which will give us the greatest ability to differentiate formulation characteristics. We have not felt it is practical to exceed 10 liters of medium and thus we have had to settle for incomplete dissolution as

final values because the saturation solubility of WR171,669·HCl is less than 250 mg/10 liters at pH 3.

Even though dissolution studies in large volumes of media are unusual they are not without precedent. Dissolution studies have been reported for griseofulvin employing volumes of 18 to 20 liters of simulated intestinal fluid (3, 4, 5). Linear correlations of bioavailability with dissolution parameters obtained in these systems were obtained.

Conclusions

The capsule formulation of WR171,669·HCl meets all compendial requirements for capsules and has a reasonably low disintegration time (i.e., < 5 minutes). Its dissolution, although incomplete in this test system, does not appear to be formulation related but rather is due to the poor solubility of WR171,669·HCl itself.

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APPENDIX A

Manufacturing Formula and Quality Control Tests on WR171,669·HCl (Halofantrine Hydrochloride) Capsules

University of Iowa College of Pharmacy

Form CP 1
1507

MANUFACTURING FORMULA

Product WR 171669AD Capsules 250 mg	List No. WRA-1
Formula Written by P Green Date 3/16/81 Formula Checked by [illegible] Date 3/18/81	Batch Size 1500
Production authorized by J F Fink	Control No. WRA-1-03181

Analysis

Assay for	Theoretical	Actual
WR 171669 AD	250 mg	250.9 mg

Control Assay No. MS 381-35 Worksheet Checked by DRT Date 3/29/81

Specifications

	Initial	Theoretical	Actual
Size	[illegible]	#0 Capsules	# 0 Capsules
Weight	[illegible]	347.5 ± 35 mg	344.5 mg
Color	[illegible]	Body: White Op. 999 Cap: White Op. 999	Body: White Op. 999 Cap: White Op. 999
Disintegration (see attached sheet)	[illegible]	NMT 15 minutes	4 minutes
Tablet Hardness			
Tablet Thickness			
Clarity			
pH			
Density			
Viscosity			
Sedimentation			
Cream Appearance			
Sterility			
Pyrogen			
Other Dissolution Test (see attached sheet)			
Wt. Variation (see attached sheet)	[illegible]	USP	USP

Package and Label

Type of Container Amber Glass vials * with standard
Size of Container 11 dram closures
Method of Packaging

Using Drug-O-Matic automatic filling machine

Remarks

* Owens-Illinois (FW-4011)

Packaged by [illegible] 3.18.81

WR 171669 HCL AD
HALOFANTRINE HYDROCHLORIDE
250 MG/CAPSULE
20 CAPSULES/BOTTLE
LOT NO.: WRA-1-03181 BOTTLE NO.:
MANUFACTURE DATE: 3/18/81
CAUTION: NEW DRUG. LIMITED BY FEDERAL LAW TO INVESTIGATIONAL USE ONLY. PREPARED FOR: WALTER REED ARMY INSTITUTE OF RESEARCH BY:

Pharmaceutical Services • College of Pharmacy
The University of Iowa • Iowa City, Iowa 52242

Product WR171669AD Capsules 250 mg List No. WRA-1

Batch Size 1500 Control No. WRA-1-03181

Caution or Special Instructions

1507

EACH CONTAINS	INGREDIENTS AND DIRECTIONS	RAW MAT'L CONTROL NO.	INITIAL		AMOUNT PER BATCH
250 mg	1. Weigh 375 gm. WR171669AD.	Army (A&C-W100-3) Lot # [illegible]707	[illegible]	[illegible]	375 gm.
7.5 mg	2. Weigh 11.25 gm. Pluronic L101 and dissolve in sufficient absolute alcohol to make a total volume of 115 ml.	BASF Wyandotte Lot # WPIG-F48A	[illegible]	[illegible]	11.25 gm.
	3. Incorporate Pluronic L101 solution into WR171669AD.		[illegible]	[illegible]	
	4. Dry the mixture at 55°C for 4 hours.		[illegible]	[illegible]	
	5. Pass the dried mixture through a #20 screen.		[illegible]	[illegible]	
90 mg.	6. Weigh 135 gm. of corn starch and mix it well with dried powder (5).	Staley A.E Lot # G 73781	[illegible]	[illegible]	135 gm.
	7. Pass the powder mixture through #20 screen.		[illegible]	[illegible]	
	8. Fill #0 capsules using DelTay Capsule Filling machine.	Parke. Davis Lot # TD 0491-2	[illegible]	[illegible]	
	9. Use 8.28 gm. powder /24 capsules. (Weigh 10 capsules for each run)		[illegible]	[illegible]	
	10. Send 30 capsules to quality control for assay.		[illegible]	[illegible]	
	Yield: 1488 Capsules		[illegible]	[illegible]	
	# of retain samples = 40				
	# of Capsules for Q.C = 30				
	Remaining Capsules = 1448				

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Final Assay of WR171,669·HCl Capsule Formulation

10 Capsules were assayed with individual assay values being:

237.9 mg	250.4 mg
266.1 mg	237.2 mg
261.3 mg	261.8 mg
263.9 mg	230.4 mg
251.4 mg	249.0 mg

Average Capsule Assay = 250.9 mg

Labelled Content = 250.0 mg

% of Labelled Content = $\frac{250.9}{250.0}$ x 100% = 100.4%

Lowest Capsule Assay = $\frac{230.4}{250.0}$ x 100% = 92.2% of label

Highest Capsule Assay = $\frac{266.1}{250.0}$ x 100% = 106.4% of label

USP XX Specifications permit a variation range of 85 - 115% of labelled content

Assay Method:

1. Each capsule was emptied into a 100 ml volumetric flask, the empty capsule shell was washed with methanol and the washings added to the 100 ml volumetric flask
2. The volumetric flask contents were dissolved by diluting to 100 ml with anhydrous methanol
3. After insoluble excipients settled, one (1) ml was pipetted from the volumetric flasked and diluted to 250 ml in a 250 ml volumetric flask
4. The resulting methanol solution was assayed by measuring the absorbance in 0.2 centimeter quartz cuvettes at 258 nm with a Pye-Unicam Model 8-100 Spectrophotometer
5. Total capsule content was calculated according to the following equation:

$$\text{Capsule content (mg)} = \frac{A \times (d.f.) \times (M.W.) \times (0.1)}{(0.2) \times \varepsilon}$$

where

A = absorbance at 258 nm

M.W. = molecular weight of WR171,669·HCl (536.9 Gm/mole)

ε = molar absorptivity at 258 nm (30,301.4) in anhydrous methanol

d.f. = dilution factor (50)

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Weight Variation of Finished Capsules
Lot No.: WRA-1-03181

No.	Mg/Capsule	No.	Mg/Capsule
1	345	11	337
2	319	12	352
3	371	13	351
4	332	14	332
5	364	15	335
6	360	16	350
7	350	17	349
8	337	18	353
9	348	19	346
10	339	20	321

Average: 344.5 mg./Capsule
Deviation from low: 7.4%
Deviation from high: 7.6%

Balance was tared with empty capsule.

BG

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1947

Disintegration Test

Item: WR 171669AD Capsules

Lot No.: WRA-1-03181

Apparatus: USP XX, p 958

Temperature: 37°C

Medium: 900 ml distilled water

Test: 4 minutes (Average of six determinations)

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Dissolution Test

Conditions:

Temperature - 37°C

Medium - 0.001 M Hydrochloric Acid (pH 3)

Medium Volume - 10 liters

Agitation - Paddle stirrer driven by a Cole-Palmer Model 4555 Variable Speed Motor

Agitation Intensity - 120 rpm

Sample Volume - 5 ml

Assay Method - UV analysis at 258 nm (molar absorptivity = 48,400)

Test Results:

Time (hr)	% Dissolved (Capsule 1)	% Dissolved (Capsule 2)
0.25	23.0	19.4
0.50	26.8	26.4
1.00	37.2	36.9
2.00	45.8	45.9
3.00	47.7	51.7
Final (>20 hours)	73.1	73.3

Comment: The extent of dissolution although incomplete appears typical for WR171,669·HCl solid dosage forms under these test conditions.

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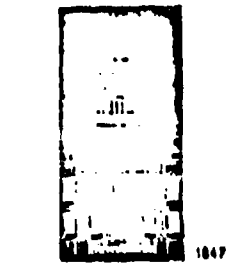

In-Process Assay of WR171,669·HCl Granulation

Amount Taken	Assay	Theoretical*	%
17.0 mg	12.7 mg	12.2 mg	105.7
21.7 mg	15.4 mg	15.6 mg	98.7
14.4 mg	10.6 mg	10.4 mg	101.9
		Average	102.1

*Based upon drug content of granulation of 71.9% (i.e., 250 mg/347.5 mg 100% = 71.9%)

Assay Method:

1. Weighed amount of granulation added to 100 ml anhydrous methanol
2. Insoluble excipients filtered off with a 0.4 micron polycarbonate filter
3. Filtrate diluted 12.5 fold with anhydrous methanol
4. Absorbance measured in 1 centimeter quartz cuvettes at 258 nm with a Pye-Unicam Model 8-100 Spectrophotometer
5. The content was calculated according to the following equation:

$$\text{Granulation Content (mg)} = \frac{A \times (d.f.) \times (M.W.) \times (0.1 \text{ liter})}{\varepsilon}$$

where

A = absorbance at 258 nm

M.W. = molecular weight of WR171,669·HCl (536.9 Gm/mole)

ε = molar absorptivity at 258 nm (50,501.4) in anhydrous methanol

d.f. = dilution factor (12.5)

BASF Wyandotte Corporation

Post Office Box 2 12
Washington, New Jersey 07882
201 689 2500

ATTENTION: Mr. John Jordan

ANALYSIS OF Pluronic L-101 Polyol

SHIPPED TO University of Iowa Iowa City, IA

DATE SHIPPED _____ CUSTOMER'S ORDER NO. _____

CAR OR TRUCK NO. _____ /WEIGHT _____

LOT NUMBER WPIB-548A

HYDROXYL NUMBER	29.1
MOLECULAR WEIGHT	
PH, AQUEOUS	6.9
CLOUD POINT	11.0
% TRANSMISSION	
VISCOSITY, cps	
FOREIGN MATTER	None
ODOR	Approved
APHA COLOR	10
% WATER	.03
ACID CORR.	

Thank you for [illegible] BASF Wyandotte for your [illegible]

Y-031-092

mw 3800

[illegible]

1720 D. R. Berg
File

Mrs. Mary Hansen
University of Iowa
College of Pharmacy
Iowa City, Iowa 52244

STA-Rx Starch USP

G-73781

6/6/79

Moisture, %	11.5
Iron - PPM	0
Ash, %	.06
pH	6.2
Color	2.7
Flavor	OK
Odor	OK
Screen Analysis:	
On #80(%)	.92
On #325 (%)	.93
Sulphur Dioxide, PPM	21.0
Oxidizing Substance	OK
FM	2.0
Standard Plate Count	40.0
Mold/Gram	0
Yeast/Gram	0
Salmonella	Neg.
E. coli	Neg.
Pseudomonas aeruginosa	Neg.
Coag. Pos. Staph. Species	Neg.

S. Martin

Product: STA-Rx 1500 starch

Manufacturer: Staley Manufacturing Co.
Lot G-73781

Identification Tests:

a) Heat with hot water: Translucent and jelly

b) Iodine Test: Positive

Result: Meets USP requirements

Control No. PG-117-059

AAPER Alcohol and Chemical Company

April 24, 1981

University of Iowa
College of Pharmacy
Iowa City, Iowa 52242

ATTN: Mr. Steve Slezak, Pharmaceutical Services

Dear Mr. Slezak:

This is to certify that the alcohol 190 proof shipped against your order # V-53713 for 5-gallon containers and gallon glass containers packaged by AAPER Alcohol and Chemical Co. from transport 734 shipped Feb. 3, 1981, conforms to all the required tests for USP Grade Ethyl Alcohol (United States Pharmacopeia, 20th Edition, dated July 1980).

This also certifies that the alcohol 200 proof shipped against your order # V-53713 for 5-gallon containers and gallon glass containers packaged by AAPER Alcohol and Chemical Co. from transport 734 shipped Dec. 11, 1980, conforms to all the required tests for USP Grade Ethyl Alcohol (United States Pharmacopeia, 20th Edition, dated July 1980).

Sincerely,

Eugene F. Hoffman
President

EFH/ds

P.O. Box 43049 • Louisville, Ky. 40243 • Telephone 502/423-0655